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A RINGWORM EPIZOOTIC IN MICE.

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THE following account of a severe infection of trichophytosis, or large-spored ringworm, which broke out at the beginning of last November amongst our breeding stock of approximately 2500 mice should be of interest to those workers who have laboratory animals under their care.

COURSE OF THE EPIZOOTIC.

The onset of the epizootic was explosive, about 400 mice developing signs of the disease within a few days of each other. Lesions which consisted typically of bald patches with inflammatory thickening or incrustation of the skin and scaliness appeared most frequently on the neck, but were common on the back and rump; the abdomen was seldom affected. The hair at the margins of the lesions had lost its lustre, was brittle and easily pulled out, and in the worst cases the whole of the animal's coat had an unhealthy, "bristling" appearance.

Fifty of the most severe cases were killed, although the tendency of the infection was towards recovery. During the next six weeks there were relatively few new cases, and apparently no increase in the normally low death-rate. At the end of December, however, it was noticed that, in addition to an occasional fresh case amongst adult mice, young mice which had seemed to be free from infection during the first two to three weeks of life developed characteristic bald areas. Soon afterwards there was a recrudescence of the infection amongst adult animals. On December 30th, or seven weeks after the onset of the epizootic, over 1000 mice of all ages were affected, and, in view of the wide dissemination and chronicity of the condition, the entire stock of nearly 2500 mice was killed.

The only three laboratory attendants who were in daily contact with large numbers of diseased animals developed the infection. Two had one lesion only, in one case on the finger and in the other on the wrist; the third case was more severe, and had a succession of lesions on hands, wrists and forehead.

The first sign of infection was usually an irritating papular erythema, which, unless promptly treated, was soon replaced by a pale red, raised and swollen and obviously inflammatory patch, with a few pustules at the mouths of hair-follicles. Treatment was satisfactory, but rather protracted, and consisted mainly of chrysarobin ointment applied at intervals for several weeks. A fourth human case occurred some weeks later in a boy who was a contact of the other laboratory attendants, and who had a typical inflammatory patch on the back of his neck. Cure was effected in about two months.

A ringworm of a similar type was discovered in four of several hundred mice from a different stock and housed in a different department of the laboratories, and in two horses in our stables which developed a number of small lesions.

LABORATORY INVESTIGATION.

For diagnostic purposes hairs from the periphery of the lesions on the mice were pulled out and placed on a slide in 10 per cent. caustic potash solution. A cover-slip was applied, and the preparation was examined under a 1/6th objective at intervals up to two to three hours. Arrangement of the spores in chains in the medulla of the hairs suggested an infection of the trichophyton group. The individual spores were oblong or square in shape with rounded corners, and were from 3 to 6μ in diameter. A similar fungus was observed in preparations of hairs from the horse and scales and pus from human lesions.

Cultivation was carried out successfully from mouse, horse and human lesions on Sabouraud's maltose agar, growth appearing about the third day as tufts, which developed typically into white discs with a central opaque mass and a large powdery areola. The reverse side of the growth was brownish red in colour. The chalky or plaster-like appearance of the colonies enabled Dr. Whitfield, who was called in consultation, to make a diagnosis of *T. gypseum* of the endo-ectothrix microides group. In later subcultures the margins of the growth presented numerous needle-like prolongations, which radiated like the rays of a star, and the strain was finally identified as *T. gypseum asteroides*.

In a few experiments on transmission and immunity, healthy mice of various ages were either housed with diseased animals, or were inoculated with cultures which were rubbed into depilated and often scarified skin. During three to four weeks of observation only 2 of 30 animals developed lesions, although a much higher incidence of infection had been expected. At this stage the experiments were rather abruptly terminated in view of the infectiousness of the disease for human subjects. Although every precaution was taken, the attendant who was looking after these mice developed a typical lesion on his wrist.

DISCUSSION.

Ringworm epizootics would appear to be much less common in mice than in other animals, for there is practically no mention of them in the literature, although serious outbreaks due to the various varieties of trichophyton have been reported in horses, cattle, dogs and man. Du Bois (1929), however, has

published an account of an epizootic in which five out of nine mice were infected, the causal fungus being *T. gypseum granulatum*. The closely related variety, *T. gypseum asteroides*, which was the cause of the present outbreak, has also given rise to a suppurating folliculitis in the horse, and has been described as a cause of large-spored ringworm in man. Amongst 67 human cases of endo-ectothrix infections, Sabouraud (1910) has met it 18 times.

The occurrence of a sudden and widespread epizootic in mice might be explained either by the introduction of a virulent infection from an outside source, or the lighting up of an infection latent in the laboratory. In the present instance the origin of the infection has not been satisfactorily traced. Two thousand of the breeding stock of mice had been received, six weeks before the outbreak, from an Institute in London which was, and still is, apparently, free from the disease. On the other hand, of six mice sent from that Institute to a house in Beckenham as children's pets, one developed two months later a typical lesion from which the *T. asteroides* fungus was cultivated.

According to Král, Wälsch, Maiocchi and Marianelli (quoted by Hutyra and Marek, 1926), the varied forms of microsporium and trichophyton are degenerations of one and the same fungus which have acquired differing properties from the different hosts upon which they have lived for several generations; under favourable conditions they may revert to their original form. This hypothesis, however, has not been generally accepted, although it may be noted that Sabouraud and Bodin are inclined to believe that the trichophyton fungus is so adaptable that it thrives free in nature on different plants, and that animals are liable to infection on coming in contact with them. Our experience suggests that it is certainly widely disseminated; some of our mouse attendants have been treated for tinea in previous years, which indicates that a variety of ringworm is frequently present in mice, though often latent and unnoticed. More recently a careful examination of several hundreds of mice in other departments of the laboratories has revealed four cases of *T. asteroides* infection, and it has also been discovered in two of our horses.

The fact that the disease appeared within three to four weeks in only 2 of 30 mice used for experiments on transmission is in accordance with the view that, while infection does occur, mice normally offer a considerable resistance. It must not be overlooked, of course, that strains of *T. asteroides* undergo considerable variations in virulence, and that the epizootic may have reached its peak before these experiments were begun.

Emphasis has been laid by some authors on the importance of predisposing factors in the transmission of trichophytosis in general. Such a factor might have been supplied in this case in the environment of the animals. The breeding mice which were originally attacked and the six animals used as pets were housed in boxes which had been recently immersed in creosote. It is possible that some degree of skin irritation or excoriation was set up which lowered the animals' resistance and assisted the localization of the fungus. Four hundred mice received from the same Institute in London five months before the onset of the epizootic were housed in tins in the same breeding-shed. They did not, of course, come in contact with creosote. When the epizootic broke out they were immediately removed to other quarters and no case of ringworm has been observed amongst them.

SUMMARY.

An epizootic is described in which nearly 50 per cent. of a breeding stock of 2500 mice were infected with *Trichophyton gypseum asteroides*.

The disease spread to four laboratory attendants.

Epizootics in mice are rare, although individual animals not infrequently become infected. It is suggested that some unusual predisposing factor or factors have determined the virulence of the present outbreak.

We wish to thank Dr. Arthur Whitfield for much helpful criticism and advice.

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COMPARATIVE TEST OF THE ESTIMATION OF HÆMOGLOBIN BY THE FLICKER HÆMOGLOBINOMETER AND THE OXYGEN CAPACITY METHOD.

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The flicker hæmoglobinometer has been described by one of us (Jenkins, 1930), and as the results obtained in routine clinical work appeared to give the colour indices an increased diagnostic value, it was determined to test the instrument against the oxygen capacity method by means of the Van Slyke apparatus, and using the technique described by Van Slyke and Stadie (1921).

The graph of the flicker instrument was calibrated to the other upon the results of a few preliminary determinations. The flicker hæmoglobinometer was as originally described, except that recently a head-rest has been added to facilitate steady inspection of the field, as the balance can be upset by a head movement equivalent to about 40 minutes of arc in the axis of the viewing telescope.

It was found necessary to calibrate the blood pipettes for the gas analysis,

as the higher viscosity of oxalated blood causes a smaller delivery than that obtained with distilled water. The following volumes were ascertained, taking the density of mercury at 15° C. to be 13·590, and of distilled water 0·998.

	Pipette No. 1.	No. 2.
Capacity volume .	1·946 c.c. .	2·405 c.c.
Delivery volumes :		
Distilled water .	1·870 „ .	2·335 „
Oxalated blood .	1·790 „ .	2·224 „

The problem of levelling the mercury before reading the volume was met by utilizing the theoretically correct method. The levels were made approximately the same—no great care is necessary—and the position of the meniscus noted. The upper tap was then opened and the gas thus placed at atmospheric pressure. If the meniscus moved the new level was noted, and its distance from the former level measured in millimetres. If the meniscus rose, denoting that the gas was under positive pressure, the distance was added to the barometric pressure ; if it fell, the distance was subtracted. Differences in level of 30 or 40 mm. cause no error by this method.

The 2 per cent. CO-hæmoglobin for the flicker determination was prepared by filling with distilled water a pipette, tested for capacity, to the mark 4·9 c.c., then delivering the water into a small dry bottle. The pipette, still holding a drop or two of water, was laid aside for a moment, whilst 0·1 c.c. of oxalated blood from another capacity calibrated pipette was blown into the bottle and the water caused to rise and fall a few times in the pipette, in order that the concentration in the bottle and the pipette might be equalized. The large pipette was then washed in and out with the hæmoglobin solution in order to add the water it contained to the solution. It is not necessary to wash out the blood pipette once again as the difference involved is too minute to be detected. To the solution before gassing about 0·01 grm. of powdered kaolin was added ; the amount need not be exact. The powder assists the precipitation of the particles in the centrifuge, which is the only means of obtaining a perfectly transparent solution. The addition of weak alkali does not give a transparent solution under critical illumination, in spite of a widespread belief to the contrary. If the turbidity were the same for all samples the point would be unimportant, but the fact has not been proved, and the average turbidity is equivalent to about 14 per cent. of hæmoglobin in its heterochromatic photo-absorption. The other details of preparation are as originally described.

The " half-strength " test, originally devised as a routine check for the flicker haemoglobinometer, was applied at frequent intervals on both instruments, and the results of it are set out in a separate table. The test consists in first estimating the full strength of the sample, then diluting the solution to exactly half strength and again estimating the sample thus diluted. The actual reading obtained is compared with the theoretical half-strength value, assuming the full-strength value to be correct. It is a rigorous test, and is probably the best single test of the inherent sensitivity of any instrument designed for quantitative analysis. For the Van Slyke apparatus

each sample was diluted with its own plasma obtained by centrifugalizing a portion of the sample. Differences in gas absorption would preclude the use of physiological saline solution. In addition to the estimation of the full-strength solution and its dilution to half strength, the plasma-diluted sample was also examined by the flicker method, as it afforded a check on the accuracy of the plasma-diluted blood supplied to the Van Slyke apparatus. This last point did not at first occur to us, and is the explanation of its omission in the first three samples in Table II.

The samples were taken at random as the opportunity arose, and are a strictly consecutive series.

TABLE I.—*Hæmoglobin in a Series of Bloods, Expressed as Oxygen Capacity (c.c. of oxygen per 100 c.c.), Measured by Oxygen Capacity and Flicker Methods.*

Oxygen capacity.	Flicker.	Discrepancy.
19·89	19·84	−0·05
20·13	21·09	+0·96
19·61	19·24	−0·37
18·50	18·14	−0·36
18·18	18·04	−0·14
17·87	17·86	−0·01
15·93	15·91	−0·02
11·09	10·83	−0·28
11·23	10·94	−0·29
24·79	25·24	+0·45
21·96	22·20	+0·24
18·40	18·22	−0·18
12·90	12·33	−0·57
15·44	15·59	+0·15
17·58	17·56	+0·18
19·40	19·04	−0·36
22·20	22·50	+0·30
18·67	18·32	−0·35
21·73	22·04	+0·31
22·70	22·33	−0·37
19·94	20·43	+0·49
17·05	16·78	−0·27
20·69	20·79	+0·10
18·74	18·96	+0·22
19·39	19·50	+0·11
21·71	21·60	−0·11
20·65	21·29	+0·62
15·24	15·46	+0·22
8·29	8·38	+0·09
16·38	16·47	+0·09
8·29	8·53	+0·24
11·15	10·92	−0·23
18·89	18·69	−0·20

Oxygen capacity.	Flicker.	Discrepancy.
12·47	12·22	—0·25
14·43	14·89	+0·46
17·86	19·05	+1·19
14·03	13·63	—0·40
16·33	16·00	—0·33
9·80	9·35	—0·45
17·24	17·30	+0·06
25·18	26·09	+0·91
15·63	15·36	—0·27
25·99	25·53	—0·46

In every instance where the discrepancy exceeded 0·50 c.c. the flicker estimation was repeated and the same result obtained.

Analysis of results :

Total number of full-strength estimations	43
Discrepancy not exceeding 0·25 c.c.	20
,, ,, ,, 0·50 ,,	38
,, ,, ,, 0·75 ,,	40
,, ,, ,, 1·00 ,,	42

The remaining result, having an apparent error of 1·19 c.c., should be examined in Table II, which seems to indicate an error in the estimation of the full-strength oxygen capacity reading. The last ten estimations are now given with their half-strength results in Table II :

TABLE II.—*Hæmoglobin, Expressed as Oxygen Capacity (c.c. of oxygen per 100 c.c.) in Full-strength and Half-strength Bloods, Measured by Oxygen Capacity and Flicker Methods.*

Oxygen capacity strengths.			Flicker strengths.			
Full.	Half.	Error.	Full.	Half.	Error.	Plasma-diluted oxalated blood.
12·47	6·09	—0·14	12·22	6·07	—0·04	..
14·03	7·74	+0·72	13·63	6·73	—0·08	..
17·86	9·55	+0·62	19·05	9·62	+0·10	..
18·89	9·66	+0·21	18·69	9·40	+0·06	9·34
14·43	6·80	—0·41	14·89	7·49	+0·05	7·49
16·33	8·16	—0·01	16·00	7·85	—0·15	7·91
9·80	5·51	+0·61	9·35	4·99	+0·32	5·20
17·24	9·41	+0·79	17·30	8·70	+0·05	8·70
15·63	8·70	+0·87	15·36	7·77	+0·18	7·96
25·99	13·14	+0·14	25·53	12·76	0·00	12·76

It will be seen that the errors of the flicker hæmoglobinometer are appreciably less in size than those of the oxygen capacity method, and that the small differences between the strength of the diluted full-strength solution of

flicker instrument and the flicker estimated strength of the plasma-diluted blood are much too small to account for the gas analysis errors.

DISCUSSION.

The two methods of examination are not strictly comparable. The gas analysis determines directly an absolute quantity, whilst the hæmoglobinometer is limited to the measurement of all samples relative to a standard that has been determined by another instrument—in this case the Van Slyke apparatus—consequently the absolute error of the latter is also an error of the comparative instrument. But in the determination of relative values the half-strength test demonstrates that the hæmoglobinometer is more accurate than the other, therefore precision is more likely to be obtained with any random sample by the use of the flicker instrument calibrated to a standard, the gas capacity of which has been carefully determined.

This last requirement necessitates a close examination of the discrepancy column in Table I. There are a few anomalous results, but the general tendency is for the flicker readings to exceed those of the gas analysis in the higher volumes and to be less than the latter in the lower range. Between 19 c.c. and 21 c.c. the frequency of the plus and minus discrepancies is approximately equal. We are not concerned to investigate this question except to suggest that the alteration in sign may be due to the use of a fixed quantity—2·1 c.c.—as the allowance to be made for physically dissolved gases. The higher range of the hæmoglobin values involves a larger number of erythrocytes and a correspondingly diminished volume of plasma. In the lower range the converse would hold.

With regard to the estimation of methæmoglobin the two methods are complementary. The oxygen method gives no indication of the presence of methæmoglobin, whilst the flicker hæmoglobinometer measures the total without distinguishing between hæmoglobin and the other substance. If it be granted that the expectation of maximum error for the gas apparatus is 3 per cent. and for the other 2 per cent., then an excess of more than 5 per cent. in the flicker reading would indicate the presence of methæmoglobin.

In the original paper describing the hæmoglobinometer it was stated that the cobalt chloride equivalent of Haldane's standard was a 20·4 per cent. cold aqueous solution of the salt. The value was obtained by pooling the contents of five tubes of the Haldane standard which were obtained at different times. The solution was filtered through a Jenkins filter and the value determined experimentally. We have found that this standard of cobalt chloride corresponds to 98 per cent. in the Haldane scale, according to the values obtained with our present gas analyses.

CONCLUSIONS.

The standard for the flicker hæmoglobinometer should be prepared by pooling samples, with appropriate dilution, taken from six or eight different individuals, each having a known oxygen capacity between 19 c.c. and 21 c.c.

In this way the fluctuating discrepancies may be expected to balance themselves out.

The half-strength test demonstrates that the oxygen capacity method employed is not as accurate as the flicker hæmoglobinometer, and that the expectation of normal error with the latter instrument is less than 1 per cent.

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SOME OBSERVATIONS ON THE DISTRIBUTION OF ANTITOXINS IN SERUM.

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IT has long been recognized that antitoxins produced by active immunization of horses with the toxins of *C. diphtheriæ*, *B. tetani* and certain other organisms are associated with the pseudo-globulin fraction of the serum. Pseudo-globulin is generally regarded as differing from the other serum proteins in the following characteristics :

1. It is not precipitated on ordinary dialysis.
2. It is not precipitated by full saturation with sodium chloride at the neutral point, though precipitation may occur if the reaction is made acid ; and—
3. Though soluble at one-third saturation with ammonium sulphate, it is precipitated by half saturation with this salt.

The fraction separating between one-third and half saturation with ammonium sulphate, however, contains some euglobulin, *i. e.* water-insoluble globulin, and similarly the precipitate obtained by one-third saturation contains antitoxin.

In a number of experiments we have found that the least quantity of ammonium sulphate which will cause precipitation of protein from a serum also precipitates traces of antitoxin ; the amount separating varies in samples from different horses. These facts illustrate the limitations of salting-out methods when applied to the preparation, in a pure state, of any particular serum protein. Such methods are, however, of great value for the purpose of comparing the distribution of antibodies in immune sera, and in this way individual variations in the sera of animals of the same and different species may be traced.

It is well known that during immunization with diphtheria toxin the total protein content of horse-serum increases, and that this phenomenon is due to an increase in the globulin fraction. Thus Ledingham (1907) reported an increase in both globulin fractions, while other workers—Gibson and Banzhaf (1910), Reymann (1924) and Kirkbride and Murdick (1927)—are agreed that the increase occurs chiefly in the pseudo-globulin fraction.

We have repeatedly noticed that different quantities of ammonium sulphate are required to precipitate the same proportions of the total antitoxin from different sera. This may be due to variations in the relative amounts of the constituent proteins.

In the experiments to be described we have attempted to determine the extent to which variations in the relative proportions of the serum proteins affect the distribution of antitoxin as determined by salting-out methods, and whether the precipitation range of antitoxin is changed on blending sera which exhibits dissimilar characteristics. For this purpose we have determined the amounts of antitoxin precipitated by various quantities of ammonium sulphate; in one instance sodium citrate was used as precipitant. We have found that this salt has an action similar to that of ammonium sulphate in precipitating protein; the chief difference between the two salts lies in the fact that in the presence of sodium citrate a serum dilution retains its alkalinity, whilst ammonium sulphate causes a relatively large fall in the pH of the solution. These salts were employed throughout our experiments in the solid form, and the quantities used are expressed in terms of the number of grammes of the salt added per litre of diluted serum. It appears that the disadvantages accompanying the use of saturated salt solutions are overlooked by most workers, though solid ammonium sulphate was used by Banzhaf and Gibson (1907) for the purpose of fractionating antitoxic plasma. These authors, however, expressed their salt concentrations in terms of saturation. We regard the use of saturated solutions and the expression of concentration in terms of percentage saturation as unsatisfactory, for the following reasons:

1. The solubility of most salts varies with temperature; it is therefore necessary to use a standard solution, the concentration of which, though actually constant, must vary in terms of saturation according to temperature.

2. In any operation involving the preparation of successive fractions, the protein filtrate becomes further diluted at each addition of the salt solution. In order to separate at the same total dilution precipitates at different saturation levels, it is necessary to vary the initial dilution of the serum for each level.

The use of solid salts eliminates these disadvantages, since it is possible to work at constant dilution, thus avoiding the calculations necessary when a saturated solution of the precipitant is used. The addition of such amounts of ammonium sulphate as are used to effect precipitation of part or all of the antitoxin causes a volume increase of the order of 10 per cent. the exact figure depending upon the amount added. In accurate work this should be determined and a correction applied. In the experiments dealt with in this paper we have neglected volume increases, as our figures are all comparative.

1. *The Determination of the Proportion of Antitoxin Precipitated by a Given Quantity of Precipitating Salt.*

The plasma or serum was diluted with the required volume of water, and 10 c.c. quantities of the dilution added to the required weights of precipitating salt, contained in test-tubes. After thorough mixing, the tubes were stoppered and allowed to stand for 18 hours at room temperature. The mixtures were were then divided into two groups :

(*a*) Those containing quantities of precipitant not exceeding 230 gm. per litre.

(*b*) Those containing concentrations in excess of this figure.

From each of the tubes containing less than 230 gm. per litre a definite volume of mixture was withdrawn and centrifuged. The supernatant liquids were then removed, and the precipitates washed with an aqueous solution of the precipitating salt of the corresponding strength. They were then re-centrifuged and the washing process repeated. The precipitates were finally dissolved in water to a convenient volume and tested for antitoxic content.

Those mixtures containing more than 230 gm. of precipitant per litre were filtered ; the precipitates were rejected, and a definite volume of each filtrate diluted with water to a convenient volume, and tested for antitoxin. In these samples, therefore, the proportion of antibody remaining unprecipitated is determined, and the amount present in the precipitate is found by calculation.

The division of mixtures according to the concentration of precipitant has two advantages :

(1) The specific gravity of those mixtures containing more than 230 gm. per litre does not permit a good separation by centrifuging.

(2) The addition of 220–230 gm. per litre effects precipitation of about half the total antitoxin. By determining the quantity remaining unprecipitated beyond this point greater accuracy is possible ; it is clearly simpler, for example, to distinguish between 5 and 10 per cent. than between 90 and 95 per cent.

The tests for diphtheria and *B. welchii* antitoxins were performed in guinea-pigs, using the intracutaneous method, and for tetanus antitoxin by intramuscular titrations in mice.

A number of uncompleted experiments have shown that some re-solution of protein occurs on washing precipitates with solutions of ammonium sulphate at corresponding concentration levels. It is necessary, however, to wash centrifuged precipitates for the purpose of removing that part of the supernatant liquid which is bound up in the precipitate ; this amount naturally varies according to the degree of spinning. In our experiments, which are comparative, we have assumed that the effect due to washing is identical for different mixtures containing the same quantity of precipitating salt.

2. *Redistribution of Antitoxin in the Presence of an Excess of Euglobulin.*

In the following experiment a 600-unit diphtheria antitoxic serum was used ; this was diluted with 2 volumes of 0·9 per cent. saline, and the distribution of

antitoxin determined as described, using ammonium sulphate. The experiment was repeated after addition of euglobulin solution to the diluted serum. The euglobulin was prepared from normal horse-serum free from antitoxin, by prolonged distilled water dialysis of a low fraction obtained by precipitation with sodium citrate. The product was dissolved in 1 per cent. sodium chloride and adjusted to 3 per cent. protein—a concentration approximately equal to that of the diluted serum.

Two serum-euglobulin mixtures were examined:

(*a*) 3 volumes of diluted serum + 1 volume of euglobulin solution.

(*b*) 1 volume of diluted serum + 1 volume of euglobulin solution.

TABLE I.—*Showing the Distribution of Diphtheria Antitoxin in a Serum, in the Presence and Absence of Added Euglobulin.*

Ammonium sulphate level (gm. per litre of diluted serum).	Percentage of total antitoxin contained in the precipitate.		
	Serum dilution alone.	Serum dilution + ⅓ volume euglobulin.	Serum dilution + 1 volume euglobulin.
110	no ppt.	<0·1	0·1–0·2
120	,,	0·2–0·5	0·5
130	,,	0·5–1·0	1·5
140	,,	2	2
150	under 0·04	3	4
160	1·5	6	10
170	6	10	14
180	14	16	24
190	..	20	28
200	24	36	36
210	30	44	44
220	36	56	56
250	92	90	92
260	94	94	94
270	98	97	98
280	98·5	98·5	99
290	99·5	99·5	..

The results are given in Table I, and show that in the presence of an excess of euglobulin, antitoxin is precipitated at much lower levels of ammonium sulphate concentration. Larger amounts of this salt, however, precipitate the same proportion of antitoxin, irrespective of the quantity of euglobulin present. The effect of introducing this protein, therefore, is to distribute the antitoxin over a wider area of protein in the serum, as determined by the salting-out method.

3. *Redistribution of Antitoxin in the Presence of an Excess of Serum Albumen.*

The methods adopted in the preceding section were repeated, using a 500-unit diphtheria serum at a dilution of 1 in 3. The albumen to be added

was prepared from an anti-tetanus serum free from diphtheria antitoxin, by collecting the precipitate produced between 300 gm. per litre and full saturation with ammonium sulphate. The dialysed product was diluted to 3 per cent. protein before blending with the serum. The following blends were examined :

(*a*) 3 volumes of diluted serum + 1 volume of albumen.
(*b*) 1 volume of diluted serum + 1 volume of albumen.
(*c*) 1 volume of diluted serum + 3 volumes of albumen.

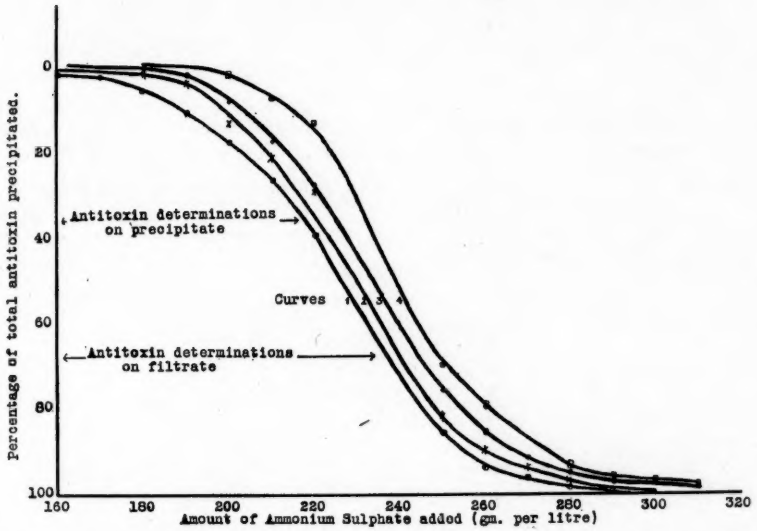

CHART I.—Showing the amount of antitoxin precipitated by various quantities of solid ammonium sulphate.
Curve 1: Diphtheria antitoxic serum dilution.
Curve 2: A mixture of 3 volumes of this dilution and 1 volume of serum albumen solution.
Curve 3: A mixture of 1 volume of the dilution and 1 volume of serum albumen solution.
Curve 4: A mixture of 1 volume of the dilution and 3 volumes of serum albumen solution.

The results are recorded graphically in Chart 1. Curve 1 shows the results obtained from tests on samples from the original serum dilution, and curves 2 to 4 those obtained from the blends *a*, *b* and *c* respectively. These curves show that the amount of ammonium sulphate required to precipitate any given percentage of antitoxin increases as the relative proportion of albumen is increased. In contrast to the behaviour of euglobulin, however, the presence of albumen does not appear to distribute antitoxin over a much wider range of serum protein.

4. *Redistribution of Antitoxin on Blending Sera.*

A. A preliminary and incomplete experiment was made to determine whether any redistribution of antitoxin occurs on blending sera. For this purpose it is necessary to blend dissimilar antitoxins; plasmas obtained from two horses immunized respectively with the toxins of *C. diphtheriæ* and *B. welchii* were therefore used. These, when examined separately at a dilution of 1 in 3, exhibited marked differences in antitoxin distribution at low levels of ammonium sulphate concentration. The dilutions were then blended in equal volumes and re-examined. The results of antitoxin tests, given in Table II, show that redistribution of both antitoxins occurred, and in any precipitate the percentage amount of each antibody, in terms of the original values, was

TABLE II.—*Showing the Distribution of Antitoxin in Plasma from Two Immune Horses and in a Blend Consisting of Equal Volumes of Each Plasma.*

Ammonium sulphate level (gm. per litre of diluted plasma).	Percentage of the total antitoxin contained in the precipitate.			
	Diluted plasmas.		Diluted blend.	
	A. Welch antitoxin.	B. Diphtheria antitoxin.	A. Welch antitoxin.	B. Diphtheria antitoxin.
140	3	..	<2	<2
150	4	<1	2	2
160	5	1½	3	2
170	6	2	4	3
180	7	6	6	6
190	..	>8	8	7
200	..	..	18	18
210	..	..	22	22

identical within the limits of the experiment; the antitoxic values of the samples were accurate to within ± 10 per cent.

B. The effect of blending on the redistribution of antitoxin was studied more closely, using three sera, A, B and C, obtained from horses immunized respectively with diphtheria, tetanus and *B. welchii* toxins. The distribution of antitoxin in each serum was investigated at a dilution of 1 in 3, using ammonium sulphate, and the figures obtained are recorded in columns 2–4 of Table III. A blend consisting of equal volumes of the three dilutions was examined in a similar manner; this contained 65 units of diphtheria antitoxin, 45 units of tetanus antitoxin and 30 units of *B. welchii* antitoxin per c.c. The figures given in columns 5–7 of Table III show that some redistribution of all antitoxins occurred. In view of the results obtained in sections 2 and 3 it appears probable that there were differences in the relative proportion of the different proteins in the three sera. On blending these proportions become averaged, and the range over which antitoxin is distributed is, consequently, altered. The results obtained from examination of the single sera indicate that A may have contained a larger proportion of euglobulin than B or C, since no workable

precipitate was obtained from either of these sera by the addition of 150 gm. of ammonium sulphate per litre ; this quantity of salt, however, was sufficient to precipitate 1 per cent. of the total antitoxin from A. If such were the case the effect of blending A with B and C would be to reduce the amount of ammonium sulphate necessary to precipitate the first traces of antitoxin from these two sera ; the figures in Table III show that this actually occurred. It will be seen that in certain precipitates obtained from the " blend serum " the proportions of each antitoxin are almost identical ; this is especially evident at the beginning and end of the precipitation range. We do not conclude from this that such figures could be obtained from any random blend, though it is to be expected that the first precipitate obtainable would contain

TABLE III.—*Showing the Distribution of Antitoxin in Serum from Three Immune Horses and in a Blend Consisting of Equal Volumes of Each Serum.*

Ammonium sulphate level (gm. per litre of diluted serum).	Percentage of the total antitoxin contained in the precipitate.					
	Diluted sera.			Diluted blend.		
	A. Diphtheria antitoxin.	B. Tetanus antitoxin.	C. Welch antitoxin.	A. Diphtheria antitoxin.	B. Tetanus antitoxin.	C. Welch antitoxin.
140	0·04–0·1	..	..	..	..	..
150	1	(no ppt.)	(negligible ppt.)	traces	traces	traces
160	2	<0·3	<1	2	<3	<3
170	7	1	<2	4	4	4
180	16	..	7	12	9	9
190	..	8	16	20	18	18
200	35	14	21	32	25	30
210	40	31	38	40	44	41
220	..	47	..	68	57	53
230	..	..	..	80	76	71
250	..	..	..	..	91	91
260	..	..	..	94	95	96
270	..	..	..	<98	<98	<98

traces of each type of antitoxin, the proportion of which would not necessarily be identical. It may be possible to obtain sera in which the relative proportions of the various proteins are so different that on blending, the antitoxins would be distributed over the same range as measured by salting-out, but separate out in entirely different proportions. Such a phenomenon occurs, as we show in section 5, when antitoxic globulin fractions are removed at different levels of salt concentration and subsequently blended.

5. *Redistribution of Antitoxin on Blending Serum Fractions.*

The effect of blending fractions was investigated, using two globulin fractions removed respectively from diphtheria and *B. welchii* antitoxic sera at different salting-out levels. The first of these, *A*, was precipitated from *B. welchii* antiserum at a dilution of 1 in 3, by the addition of 240 gm. of

sodium citrate per litre of diluted material. The precipitate was washed with a solution of sodium citrate of corresponding strength, pressed and dialysed. The second fraction, *B*, was prepared from anti-diphtheria serum by collecting the precipitate formed between 240 and 330 gm. of sodium citrate per litre. The fractions after dialysis were diluted to 3 per cent. protein, 0·3 sodium chloride and 0·2 per cent. tricresol ether added. Fraction *A* contained 90 units of *B. welchii* antitoxin and *B* contained 400 units of diphtheria antitoxin per c.c. The distribution of antibody in each globulin solution was determined

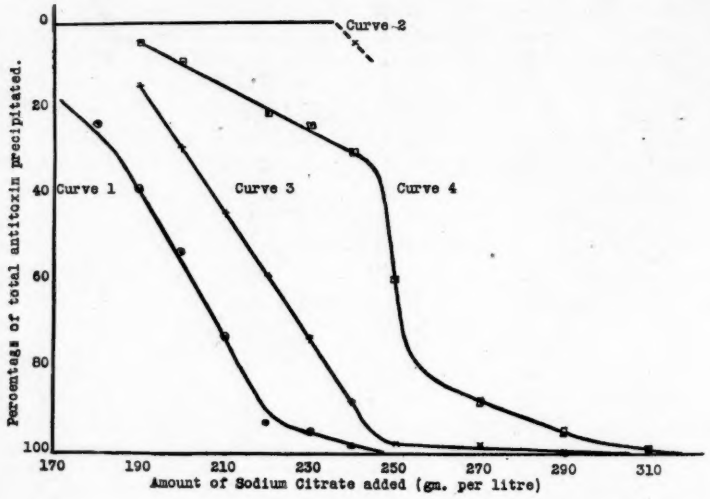

CHART II.—Showing the amount of antitoxin precipitated by various quantities of solid sodium citrate.

Curve 1: A solution of Welch antitoxic globulin prepared by precipitation with 240 gm. of sodium citrate per litre of diluted serum. Fraction *A*.

Curve 2: A solution of diphtheria antitoxic globulin prepared by precipitation between 240 and 330 gm. of sodium citrate per litre of diluted serum. Fraction *B*.

Curve 3: A mixture of equal volumes of these two solutions (distribution of Welch antitoxin).

Curve 4: A mixture of equal volumes of these two solutions (distribution of diphtheria antitoxin).

by re-precipitation with sodium citrate, and the results are recorded graphically in Chart 2, curves 1 and 2. It will be seen from curve 1 that practically no readjustment of precipitation levels occurred on removing fraction *A*, 98 per cent. of the total antitoxin still being precipitated from the fraction on adding 240 gm. of sodium citrate per litre. Owing to a lack of sufficient material, we were unable to trace the course of precipitation of antitoxin from fraction *B*; it will be seen, however, from Curve 2 that no detectable antibody was precipitated by amounts of salt less than 240 gm. per litre.

A blend consisting of equal volumes of the two fractions was subsequently

examined by determining the proportion of each antitoxin separating on addition of various quantities of sodium citrate. The results are given in curves 3 and 4 in Chart II, where curve 3 shows the proportions of antitoxin from fraction *A* and curve 4 those from fraction *B*. It will be seen that on blending, considerable redistribution of both antitoxins occurred, but although all the precipitates examined contained easily detectable amounts of both types of antibody, the proportions of these were by no means the same ; as would be expected, the maximum differences are encountered in the central part of the range. We therefore conclude that pseudo-globulin fractions separated by precipitation with relatively high concentrations of salt, exert an influence on lower fractions similar to that of albumen on a whole serum. The action of the low fraction *A* resembles that of euglobulin, but appears to be more extensive : 24 per cent. of the total antitoxin from fraction *B* is present in the precipitate at 230 gm., whereas this quantity of salt was insufficient to cause any precipitation from the unblended fraction.

It must be pointed out that although redistribution of antitoxin appears to have occurred in these experiments, the expression is not strictly justifiable. According to present theories, a solution of different types of proteins contains particles of many graded sizes existing in equilibrium, some of which are formed by association, while some are present as single molecules. Thus the particles of pseudoglobulin as found in a serum are not uniform. It may therefore be incorrect to state that " antitoxin is re-distributed on adding euglobulin to serum," for the system is altered, and in order to attain equilibrium, association or dissociation of molecular complexes may occur. Whether the expression is justified depends upon three conditions :

(*a*) The extent to which association and dissociation occur in the system.

(*b*) Whether a definite quantity of precipitating salt invariably precipitates all particles of a certain size, irrespective of the relative proportions of different proteins present in the solution ; and

(*c*) Whether there is any mutual reaction between the particles of a certain protein remaining in solution and those precipitated by the salt.

For practical purposes, however, we may state that " as determined by salting-out methods, antitoxin may be redistributed in a serum or a fraction by altering certain conditions " such as we have described.

SUMMARY.

(1) The use of solid ammonium sulphate for the purpose of fractionating antitoxic sera has many advantages over the use of saturated solutions. The quantities should be expressed in terms of grammes of the salt per litre of serum and not as percentage saturation, since this is variable, depending on the temperature.

(2) The quantity of ammonium sulphate necessary to precipitate a given proportion of antitoxin may be altered by addition to the serum of an excess of either of the non-antitoxic proteins, euglobulin or serum albumen.

(3) On blending sera which exhibit differences in antitoxin distribution as determined by salting-out methods, some redistribution of antibodies occurs.

(4) On blending two globulin fractions containing different types of antitoxin, one removed at low and the other at high concentration of precipitating salt, redistribution of the antibodies from both fractions occurs: any quantity of precipitant sufficient to cause separation of protein precipitates both types of antitoxin, though in different proportions.

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OBSERVATIONS ON THE ACIDITY AND CHLORIDE CONTENT OF THE GASTRIC JUICE.—II.

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IN any investigation of the physiology of gastric digestion, it is desirable that distinction be made between the gastric juice as secreted and the fluid that collects in the stomach during digestion. The difficulty of obtaining the former compared with the ease of collecting the latter has frequently led to the statement of conclusions regarding gastric secretion, which are based on observations made on fluid that has been lying for an indeterminate time in the stomach. It is a fact of common observation that gastric fluid may be bile-stained—an indication that duodenal fluid is being regurgitated into the stomach. This was observed in 72 per cent. of cases examined by Apperly and Norris (1930). Duodenal fluid may be made up of a mixture of bile, intestinal juice and pancreatic secretion in widely varying proportions. The regurgitation of this mixture into the stomach contents gives a fluid, the constituents of which are derived from so many sources that difficulty is experienced in tracing their origin. This has been exemplified particularly in the case of the sodium chloride content of gastric fluid. The possible sources of the sodium chloride are the gastric secretion, the duodenal fluid and the interaction of pancreatic sodium bicarbonate in the duodenal fluid, with gastric acid. It is not believed that the sodium chloride of regurgitated duodenal fluid is the source of the sodium chloride in the fluid found in the stomach. The view that sodium chloride is derived almost exclusively from gastric secretion is held by MacLean and

Griffiths (1928), who say that the slight regurgitation of alkaline fluid into the stomach which occasionally occurs plays little or no part in the regulation of gastric acidity. Morrell Roberts (1927), Hansman, Day and Clifton (1927) and Apperley and Norris (1930), while agreeing that the gastric secretion is the source of sodium chloride in gastric fluid, believe that neutralization of gastric acid by duodenal fluid is a subsidiary factor contributing to the total sodium chloride found. Bolton (1928) does not deny that sodium chloride may be present in gastric secretion, but regards the regulation of gastric acidity as a phenomenon controlled by duodenal regurgitation, and the regulating mechanism as one of neutralization. Boldereff (1929) points to neutralization as the source of gastric sodium chloride. He insists that gastric juice is always secreted in the same degree of acidity and therefore regards statements that gastric secretion may be of such a nature as to decrease the already existing acid of stomach fluid as tantamount to the postulation of the existence of two distinct kinds of gastric secretion. Denying this, and adhering to the constancy of the acidity of gastric secretion, he attributes the sodium chloride in gastric fluid to the reflux of duodenal fluid into the stomach.

The observers mentioned above have employed various methods of investigation of the questions of what is the composition of gastric secretion and what controls its acidity, or more broadly, its chloride partition between acid and sodium chloride. Conflict of opinion, however, still persists. Evidence on these questions which will be presented in this paper has been produced in an examination of gastric secretion and duodenal fluid, obtained simultaneously and continously without either one having been allowed to mix with the other.

TECHNIQUE.

The investigations were carried out on human subjects, who were prepared in the usual way for a test-meal. In order to prevent mixing of gastric secretion and duodenal fluid, two tubes were introduced in the subject simultaneously. One of these was an ordinary Ryle gastric tube marked 55 cm. from the tip, and the other was a longer " duodenal " tube with a perforated metal tip attached and marked 80 cm. from the tip. By carefully placing the tubes side by side it was found that the subject was able to swallow both nearly as readily as one. If he encountered difficulty in swallowing them, a small drink of water was allowed to be taken slowly and the tubes were fed in as he swallowed. When the tubes had been passed to the 55 cm. mark they were placed in the most comfortable position in his mouth, and he was thereupon arranged lying on his right side. The Ryle gastric tube was fixed in position by attachment to the clothing of the subject, who was instructed to swallow, at intervals of a few minutes, a short length of the duodenal tube until it had been passed to approximately the 80 cm. mark. This operation occupied about forty minutes at least in order that the desired object of passing the tube into the duodenum might be attained. Fluid was then withdrawn through the tube and tested with bromthymolblue, a blue colour being taken as an indication that the tube was in the duodenum. In these circumstances the fluid withdrawn was always markedly bile-stained. It appeared that the tubes reached the desired positions

more readily in those subjects who were allowed to drink a small quantity of water during the first stage of the swallowing. Subsequent results with those who had swallowed the tubes with the aid of water and with those who had not required water were indistinguishable. The technique described is slightly modified from that of Lim, Matheson and Schlapp (1923), who used it for investigation of the secretory function of the stomach and duodenum.

When the tubes were in proper position, the subject was allowed to sit in a comfortable posture. A 100 c.c. Erlenmeyer flask fitted with a rubber stopper through which pass two glass tubes is attached to the gastric tube and to a filter pump which is connected with a small mercury manometer, and a similar arrangement is made with the duodenal tube. In this way continuous suction may be applied to both the gastric and duodenal tubes. In the experiments here reported, continuous collection of the gastric secretion and duodenal fluid was made for ¼-hour periods, at the end of which the flasks were rapidly replaced by fresh ones for the next collection period. The filter pump was run at such a speed that it continuously maintained a reading of 5–8 cm. on the mercury manometer. Owing to variations in water pressure the pump could not be relied upon to work constantly, and so there was placed in the rubber connections a small glass T-piece with one arm closed by a rubber tube and spring clip, through which arrangement air could be allowed to enter the apparatus if the manometer reading rose above 8 cm. To prevent saliva reaching the stomach contents the usual precaution of providing a dish for the subject to spit into was taken.

When the apparatus was arranged, the pump was run for about 15 minutes to test that the tubes were freely collecting fluid, and to remove from the stomach and duodenum any water that had been swallowed and fluid that had collected under uncontrolled conditions. If the gastric tube was failing to collect the fluid in the stomach, the duodenal tube was found to collect an unusually large amount of fluid only lightly bile-stained. If the duodenal tube was not functioning, the gastric fluid usually was bile-stained. Proper working having been established, the test collection flasks were removed and immediately replaced by fresh flasks. Two ¼-hour collections were made from the stomach and duodenum. The subject then was given a subcutaneous injection of 1 mgm. of "ergamine" (approximately equivalent to 0·5 mgm. histamine) in 0·5 c.c. distilled water, and the collection of the gastric secretion and duodenal fluid was continued over ¼-hour periods. The volume of the specimens obtained was measured, and they were centrifuged in order to obtain clear specimens for analysis.

EXPERIMENTAL RESULTS.

Gastric secretion obtained before histamine injection.—It is seen in Table I that gastric juice is secreted in varying quantity and composition, and that no relation exists between the rate of secretion and the composition. The secretion in the six cases examined had a range of variation of total chloride from 0·241 to 0·436 gm. chloride ion per 100 c.c., of total acidity from 0·007 to 0·231 gm. chloride ion per 100 c.c., and of sodium chloride from 0·151 to 0·361 gm. chloride ion per 100 c.c. In the six cases examined similar analyses were encountered in Nos. 2 and 6, but this group and the other four cases differed between them-

selves in the amount of total acid, total chloride and sodium chloride in 100 c.c. of gastric secretion.

Gastric secretion obtained from the stomach stimulated by histamine.—In continuation of the same investigation in the six subjects, the specimens

TABLE I.—*Results of Examination of Gastric Secretion Collected before Stimulation of the Stomach by Histamine.*

Subject.	Specimen collected in—	Volume (c.c).	Total acidity (gm. Cl per cent.).	Total chloride (gm. Cl per cent.).	NaCl (gm. Cl (per cent.).
1	1st quarter-hour	11	0·231	0·369	0·138
	2nd ,,	8	0·224	0·375	0·151
2	1st ,,	15	0·007	0·241	0·234
	2nd ,,	25	0·010	0·266	0·256
3	1st ,,	14	0·124	0·350	0·226
4	1st ,,	16	0·064	0·251	0·187
	2nd ,,	8	0·117	0·349	0·232
5	1st ,,	14	0·089	0·400	0·311
	2nd ,,	17	0·075	0·436	0·361
6	1st ,,	4	0·021	0·254	0·233
	2nd ,,	3	0·007	0·253	0·246

TABLE II.—*Results of Examination of Gastric Secretion Collected after Stimulation of the Stomach by Histamine in the Same Six Subjects as Described in Table I.*

Subject.	Specimen collected in—	Volume (c.c.).	Total acidity (gm. Cl per cent.).	Total chloride (gm. Cl per cent.).	NaCl (gm. Cl per cent.).
1	3rd quarter-hour	38	0·316	0·471	0·155
	4th ,,	64	0·458	0·549	0·091
2	3rd ,,	31	0·110	0·366	0·256
	4th ,,	45	0·242	0·390	0·148
3	2nd ,,	30	0·352	0·520	0·168
	3rd ,,	48	0·475	0·567	0·092
	4th ,	37	0·486	0·567	0·081
4	3rd ,,	16	0·301	0·462	0·161
	4th ,,	46	0·430	0·535	0·105
5	3rd ,,	34	0·185	0·475	0·290
	4th ,,	66	0·366	0·506	0·140
	5th ,,	55	0·348	0·487	0·139
6	3rd ,,	16	0·085	0·300	0·215
	4th ,,	26	0·231	0·410	0·179
	5th ,,	37	0·316	0·453	0·137

described in Table II were collected following the administration of histamine at the end of the collection of the specimens described in Table I. The effect of histamine as a stimulant of gastric secretion is at once apparent, the volumes of secretion being all markedly increased. The total chloride has increased in

concentration whilst the sodium chloride has remained unchanged or decreased, and therefore the effect of histamine is not merely to increase the rate of gastric secretion, but also to increase its chloride content by increasing its proportion of total acid to sodium chloride. The ideas that the acidity or total chloride of gastric secretion remains constant, and that the rate of secretion governs the amounts of these substances in fluid in the stomach, are disproved by the quite appreciable variations seen in Table I and the very marked variations in Table II. Not only does the composition of the gastric secretion vary from one

TABLE III.—*Variation in Volume and Total Chloride of Duodenal Fluid before and after Histamine Injection.*

Subject.	Specimen collected in —	Volume (c.c.).	Total chloride (gm. Cl per cent.).
1	1st quarter-hour	27	0·341
	2nd ,,	12	0·392
	3rd and 4th quarter-hour	3	0·434
2	1st quarter-hour	21	0·302
	2nd ,,	10	0·433
	3rd and 4th quarter-hour	2·5	0·438
3	1st quarter-hour	10	0·285
	2nd ,,	3·5	0·406
	3rd and 4th quarter-hour	2·5	0·337
4	1st quarter-hour	20	0·417
	2nd ,,	8	0·426
	3rd and 4th quarter-hour	3·5	0·384
5	1st quarter-hour	16	0·450
	2nd ,,	9	0·427
	3rd and 4th quarter-hour	No specimen appeared.	
	5th quarter-hour	7·5	0·438
6	1st ,,	5	0·349
	2nd ,,	11	0·528
	3rd ,,	4	0·293
	4th ,,	4	0·253
	5th ,,	7	0·347

individual to another, but in any one subject the secretion alters appreciably with the slight stimulation of a gastric tube, and alters markedly with the strong stimulation of injected histamine. The constancy of the acidity or of the negative ion concentration of gastric juice cannot, therefore, be upheld.

Duodenal fluid obtained before and during gastric stimulation by histamine.—In Table III are given the results of the examination of duodenal fluid collected simultaneously with the gastric secretion which has been described in detail above in Tables I and II. The range of variation of the total chloride content of duodenal fluid remained approximately the same before or during the stimulation of gastric secretion by histamine injection. After histamine

injection, however, the quantity of duodenal fluid fell to a very small figure, which in two cases was only 2·5 c.c. for the two quarter-hour periods following the injection. A similar result has been observed by Lim, Matheson and Schlapp (1923). It is of interest to note that the specimens of duodenal fluid obtained after histamine injection were extremely deeply coloured with bile, and in one case (Subject No. 6, 5th quarter-hour specimen) the very dark viscid fluid was of neutral reaction to bromthymolblue. The quantity of pancreatic juice in the specimen was therefore negligible. Lim and Schlapp (1923) and Osborne (1928) have found in dogs that, following histamine injections, the flow of gastric fluid into the duodenum results in stimulation of the pancreas to vigorous secretion. If, however, the pylorus be ligatured to prevent gastric fluid from reaching the duodenum, an absence of pancreatic secretion is observed. In the present experiments the continuous removal of gastric secretion from the stomach stimulated by histamine gives a condition similar

TABLE IV.—*Results of a Fractional Test-Meal in which the Specimens Contained Very Large Amounts of Sodium Chloride.*

Time.	Total acidity (gm. Cl per cent.).	Total chloride (gm. Cl per cent.).	Sodium chloride (gm. Cl per cent.).	Ratio of NaCl (as Cl) to total acidity (as Cl).
Before	0·0568	0·358	0·301	5·8
20 minutes	0·0355	0·341	0·305	8·6
40 ,,	0·0213	0·337	0·316	14·8
60 ,,	0·0213	0·305	0·284	13·3
80 ,,	0·0213	0·337	0·316	14·8
105 ,,	0·0248	0·380	0·355	14·3
130 ,,	0·0639	0·390	0·326	5·1

to that established by Lim and Schlapp, and Osborne. Therefore the very small bulk of the duodenal fluid, which may be neutral to bromthymolblue, indicates that the method of continuous withdrawal of gastric secretion prevents the escape of any gastric fluid into the duodenum. Also the absence, which was noted, of any trace of bile-pigment from the gastric fluid proves that none of the biliary duodenal fluid had regurgitated into the stomach. The experimental procedure therefore functions satisfactorily in effecting the separate collection of gastric secretion and duodenal fluid, and in precluding the possibility of any admixture of the two having occurred.

DISCUSSION.

There has been difference of opinion with regard not only to the quantitative composition of gastric secretion, but also to the qualitative nature. Fluctuations in the acidity of fluid in the stomach may be caused by the addition of a more or a less acid gastric secretion, or by the regurgitation of alkaline duodenal fluid and consequent neutralization of acid. If the latter explanation were true, it would not be inconsistent with some observed facts to postulate

constancy of acidity of gastric secretion. But it has been demonstrated that gastric secretion is a fluid of inconstant composition, the total chloride, the total acidity and the ratio of total acidity to total chloride being subject to marked and rapid variation under given stimuli. In a previous publication (Rudd, 1930) the gastric secretory response to test-meals of gruel and of cream was examined. In Chart II (cream) in that paper are graphed the highest sodium chloride values that were obtained in the series. These values have now been transposed into the form shown in Table IV, where the ratio of sodium chloride expressed as grammes of chloride ion per 100 c.c. to total acidity in grammes of chloride ion per 100 c.c. has been calculated for all the readings taken throughout the test-meals. In Table I, Subject No. 2, the 1st quarter-hour specimen has a ratio, calculated similarly, of 0·234/0·007, which equals 33, and in Subject No. 6, the 2nd quarter-hour specimen has a ratio of 0·246/0·007 which equals 35. These ratios are much larger than the highest found in Table IV, which deals with a test-meal showing an extremely high sodium chloride content. Thus gastric secretion may contain a sufficiently large quantity of sodium chloride to account completely for the sodium chloride found in the fluid in the stomach during a test-meal. It is therefore unnecessary to postulate that duodenal regurgitation is the factor controlling gastric acidity, because the acidity and rate of gastric secretion and possible control of the acidity of stomach contents is a function inherent in the phenomenon of gastric secretion itself. The presence of sodium chloride as a constituent of gastric secretion is established beyond doubt. Its presence is not adventitious, for comparison of the sodium chloride with the total chloride gives a measure of the tendency of the stomach to secrete a more or a less acid juice for the control of the acidity of its contents.

Regurgitation of duodenal fluid of sufficient alkalinity into the stomach would decrease gastric acidity, and from the experimental evidence discussed in this paper it is impossible to state dogmatically that it is not an important controlling factor of gastric acidity. Information, however, is lacking concerning a precise measurement of the amount of duodenal regurgitation. It cannot be measured by the amount of biliary pigment in gastric fluid, because bile itself is present in variable amount in duodenal fluid. Its attempted measurement by MacLean and Griffiths (1928), by estimations of carbon dioxide and of trypsin in gastric fluid, indicated that only very small quantities of pancreatic juice could have mixed with fluid in the stomach, although the acidity had greatly declined. The available evidence, then, favours the view that duodenal regurgitation may occur only to a slight extent. In this paper the existence inherent in the stomach of a means of controlling its acidity is affirmed, and it does appear a more probable explanation of observed facts than that which can be offered by the phenomenon of duodenal regurgitation, which is not even susceptible to direct measurement.

The attempted elucidation of the nature of gastric secretion by test-meals or by placing in the stomach various chemical substances is beset with difficulty because several factors contribute to gastric digestion. Even when the substance placed in the stomach can be quantitatively estimated and so afford information concerning its dilution by the digestive juices, the resulting conclusions regarding the gastric secretion are applicable strictly only to the specific

conditions being studied. This objection can be made to the experiment of MacLean and Griffiths (1928), in which they placed 0·11 normal sulphate solution in the stomach, and found that as gastric secretion continued, the sum of the sulphate and chloride concentrations remained 0·11 normal. This must have been brought about by the secretion of a fluid containing 0·11 normal chloride, and therefore they conclude that this always represents the total chloride content of gastric secretion. In addition to this, it is generally agreed that the fluid in the stomach does attain an approximately constant concentration of chloride. It is incorrect, however, to interpret these results as proof that the gastric secretion always contains chloride in 0·11 normal or in any other fixed concentration. This has been demonstrated in the experiments detailed above by the variability of the total chloride, which ranged from 0·068 normal (Table I, Subject No. 2, 1st quarter-hour specimen) to 0·16 normal (Table II, Subject No. 3, 3rd and 4th quarter-hour specimens).

SUMMARY.

1. Sodium chloride is a normal constituent of gastric secretion.

2. When the secretion is stimulated by histamine, the rate and the total chloride content of the secretion are increased.

3. The total chloride content of the secretion varies widely. The variation may be due to changes in total acidity or in sodium chloride, or both.

4. The variations of acidity and sodium chloride are sufficiently large to account for the changes in acidity of stomach contents that occur.

5. It is considered that the available evidence points to the ability of the stomach to control its acidity, and that whatever neutralization occurs as a result of duodenal regurgitation is merely a secondary factor.

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A STUDY OF THE PHOSPHORUS DISTRIBUTION IN BACTERIAL CULTURES.—PART II.

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In a previous paper Gordon and Marshall (1930) record an investigation of the changes which occur in the organic, inorganic and lipin phosphorus content of media during the metabolism of various bacteria, and though the details vary quantitatively for different species, it was found that generally the lipin and organic phosphorus compounds of the medium underwent hydrolysis, with the resultant accumulation of inorganic phosphorus. In addition a preliminary estimate of the relative distribution of lipin and non-lipin phosphorus in the bacteria was made. It was suggested that in all probability this lipin phosphorus was synthesized from inorganic sources.

The organisms were grown in peptone bouillon media containing some lipin and organic phosphorus, and it seemed important to investigate the phosphorus distribution of bacteria grown in synthetic media containing only inorganic phosphorus, with a view to demonstrating the possible synthesis of organic phosphorus from this source. The present paper contains an account of such experiments using *B. coli*.

Whitehead (1928) concluded that "*inorganic* phosphorus seems to be necessary for the production of growth of streptococci in twenty-four hours, and that phosphate in some form is probably essential." Friedlein (1928) investigated the necessary concentrations of the compounds essential to promote the growth of certain species of bacteria and showed that phosphate was necessary for the growth of *B. coli*, but that too high a concentration was as inhibitory as one which is too low. We have utilized his results in devising a suitable synthetic medium, but experienced at first a difficulty in obtaining enough growth to furnish us with sufficient material for analysis. After various modifications and additions a suitable medium was obtained, though we have never been able to equal the heavy growths obtained with peptone bouillon.

EXPERIMENTAL.

The following medium was made up for each series of experiments recorded in this paper:

15 gm. of purified gelatine.
20 gm. of pure glucose.
1000 c.c. of potassium dihydrogen phosphate solution containing 0·5 mgm. of phosphorus per c.c.
5 gm. of ammonium nitrate.
2 gm. of potassium sulphate.
0·25 gm. of sodium chloride.
0·1 gm. of magnesium chloride, and
A trace of ferrous sulphate.

Made up to two litres with distilled water after adjusting the pH to 8·3 (just pink to phenolphthalein) with dilute caustic soda.

The solution was autoclaved, and if necessary adjusted to pH 7·5. It was observed that, after inoculation and incubation at 37° C. for four days, the pH dropped to 6·0. At this pH, which is well above the iso-electric point of gelatine (4·6), no combination of gelatine and phosphate would be expected to occur. The solution was perfectly clear, and did not set to a solid after the growth of the bacteria, thus permitting them to be centrifuged.

Purification of Gelatine.

The commercial " pure " gelatine was found to contain large amounts of phosphorus, but this was quite simply removed by soaking thin strips of the gelatine in 0·002 N. sulphuric acid for about two days, changing the wash liquor frequently ; then for a further day in 0·001 N. acid, and finally in distilled water until the pH of the wash water no longer changed. Two c.c. of a 3 per cent. solution contained no detectable trace of phosphorus after this treatment, so that the quantity introduced into the media from this source was less than 0·0005 mgm. per c.c.

Preliminary Experiments.

It was next necessary to determine whether any of the constituents of the medium interfered with the analytical determinations performed as described in the previous paper, and also whether the processes of autoclaving and incubating the medium led to the formation of phosphorus complexes, which would appear in the analytical results as organic or lipin phosphorus.

Rimington (1924) has shown that the presence of ammonium sulphate interferes with the colour formed in the estimation of phosphorus by Briggs's method if present in appreciable amount, though the production of ammonium salts from the nitrogen of proteins, during the phosphorus analyses of these substances, was insufficient to produce erroneous results. Our solutions contained, in addition to gelatine, added ammonium salts, and in consequence a series of experiments were made to find if interference occurred under our conditions. No difference was found provided a full half hour was allowed to elapse before the colours were read.

Analyses were next made for total, inorganic and lipin phosphorus on the medium after autoclaving and incubating for a week, and compared with an anyalsis of potassium dihydrogen phosphate solution made up to the same strength. No lipin phosphorus was found, and exactly the same results were obtained for the total and inorganic phosphorus content of both solutions (after allowing for the measured concentrating of the medium during incubation). A large number of analyses of standard solutions were made, and it was found that though the experimental error on individual determinations was about 5 per cent., two series of three or more determinations made at the same time gave average results which did not differ by more than 0·001 mgm. phosphorus per c.c.

In addition analyses of glucose and gelatine solutions 5–10 times stronger than the media were made after autoclaving and incubating with the same

concentration of phosphate at the same pH, and again no difference was found between the inorganic and total phosphorus contents. Hence it is concluded that the amount of organic phosphorus present in the medium cannot exceed 0·0005 mgm. per c.c. This quantity is definitely less than the quantities subsequently found in the bacterial cultures and given in the table (0·0011–0·0031).

It was found necessary to estimate the inorganic phosphorus by the Bell and Doisy method as in the previous paper, since gelatine is not precipitated by trichloracetic acid in the concentration used, and gives a precipitate with molybdic acid soluble in sodium carbonate.

Treatment before Analysis.

The volume of the medium was measured before and after incubation in order to allow for evaporation in those experiments in which the medium was analysed. The solutions were inoculated with *B. coli* after autoclaving and then incubated at 37° C. for four days. The bacteria were centrifuged off at a high speed, and the supernatant medium decanted and analysed along with uninoculated controls (see analyses of centrifuged media). In experiments 1, 2 and 3 (see analyses of centrifuged bacteria) the centrifuged deposits of bacteria were suspended in normal saline and re-centrifuged. After this process had been performed once or twice the bacteria were made up to 25 c.c. with water. In Experiments 4 and 5 the bacteria were not washed, but in Experiment 4 made up to 25 c.c. with water, and in Experiment 5 with the medium from which they had been centrifuged. The analysis of this medium is given in the last column of the table. The figures in the table headed " Dilution " are an approximate estimate of the number of times the original medium has been diluted by these procedures, and are inserted to show the continued decrease of inorganic phosphorus with increasing washing. Experiment 3 shows that an abnormally large amount of inorganic phosphorus was present. This can be attributed to an insufficient lixiviation by the wash-waters of the small quantity of bacteria obtained from the 800 c.c. of medium which were centrifuged. This was actually the first experiment made; in all subsequent experiments the clumps of bacteria were broken up with a glass rod to ensure complete mixing on washing.

Analyses of Bacterial Suspensions.

Total phosphorus determinations were made in triplicate on 1 or 2 c.c. (according to the amount of phosphorus) as described in the previous paper. If the oxidation with the nitric-sulphuric acid mixture did not give a colourless solution after boiling off the nitric acid, another c.c. of pure nitric acid was added and boiled off again before the final oxidation with hydrogen peroxide.

Inorganic phosphorus was determined by pipetting 5 or 10 c.c. into centrifuge tubes, and adding 1 or 2 c.c. of 25 per cent. trichloracetic acid. After mixing, these solutions were centrifuged, as it was found impossible to obtain clear solutions by filtration. After two to three hours quantities of 1, 2 or 4 c.c. were withdrawn for analysis by the Bell and Doisy method, the final figure being the mean of three or more determinations.

Lipin phosphorus determinations were made as described in the previous paper, 50 c.c. of the alcohol-ether extract being evaporated and total phosphorus determined. Analyses were made in triplicate.

TABLE.

Experiment.	1.	2.	3.	4.	5.	Medium from Expt. 5: 5a.
Volume centrifuged	1600 c.c.	1600 c.c.	800 c.c.	1400 c.c.	1600 c.c.	..
Dilution	10^6	10^5	10^3	10–50	0	..
Total phosphorus	0·148 mgm.	0·179	0·058	0·146	0·492	0·284
Inorganic ,,	0·001 ,,	0·005	0·022	0·023	0·292	0·284*
Organic ,,	0·147 ,,	0·174	0·036	0·123	0·200	..
Lipin ,,	(0·015) ,,	0·025	0·006	0·017	0·023	..
Lipin/organic per cent.	(10)	14	17	14	12	..
Organic per c.c. medium	0·0023 ,,	0·0027	0·0011	0·0022	0·0031	..

The figures in brackets are the result of a single determination ; all others are the average of three or more determinations.

* In Experiment 5 it was found that 800 c.c. of the medium had evaporated to 700 c.c. during autoclaving and incubating, so that the original concentration corresponding to this figure is $7/8 \times 0{\cdot}284 = 0{\cdot}249$. The solution was made up to a strength of 0·250 mgm. per c.c. originally.

RESULTS I : CENTRIFUGED BACTERIA.

The results given in the table are calculated for 1 c.c. of the bacterial suspension. The volume of the medium from which the bacteria were centrifuged is given in the first line. These bacteria were made up to 25 c.c. after washing as described above ; hence the analytical results for 1 c.c. of this suspension represent one twenty-fifth of the amount of phosphorus present in the total bacteria of the medium centrifuged in each experiment. The amount of organic phosphorus present in the original medium due to the bacteria can thus be calculated from the amount found in the centrifuged bacteria, and its concentration per c.c. is given in the last line. Its amount will be seen to be definitely greater than could have been present before the growth of the bacteria, and thus represents organic phosphorus which has been synthesized.

RESULTS II : CENTRIFUGED MEDIA.

A series of analyses of the centrifuged medium after the growth of *B. coli*, and of uninoculated controls, failed to show any organic or lipin phosphorus in the medium. The possibly small decrease in inorganic and in total phosphorus was on the border of experimental error.

DISCUSSION OF RESULTS.

That no changes could be found in the composition of the media after the growth of *B. coli* was a result not unexpected, first because with the growths obtained the amount of inorganic phosphorus utilized was so small as to come within the region of experimental error, and secondly because, if any organic phosphorus had been liberated into the media, it would have been rapidly hydrolysed to inorganic phosphorus, as was shown by the results of the previous paper.

It will be seen that the lipin phosphorus present in the bacteria accounts for approximately 14 per cent. of the organic phosphorus present. This quantity, if distributed through the medium from which the bacteria were centrifuged, would be undetectable, hence no direct proof of the synthesis of lipin phosphorus from inorganic sources has been obtained. On the other hand, the amount of organic phosphorus is definitely greater than was present in the original medium, and therefore some organic phosphorus must have been synthesized from inorganic phosphorus. As much as 86 per cent. of this organic phosphorus was present in a form other than lipin phosphorus.

The previous communication showed that lipin phosphorus was readily hydrolysed in the medium. In our experiments the fact that the lipin phosphorus of the bacteria occurred in definite amounts when there was none originally present in the medium, suggests that the lipin content of the bacteria is independent of the concentration of lipin phosphorus in the medium, and that the bacteria are capable of synthesizing lipin phosphorus from inorganic sources.

In experiment 5 it will be seen that the concentration of inorganic phosphorus of the bacteria is somewhat higher than in the surrounding medium. The other experiments show that this inorganic phosphorus can be almost completely removed by successive washing. It is for this reason that we have expressed the lipin phosphorus as a percentage of the organic phosphorus, since the total phosphorus diminishes on washing by reason of the removal of the inorganic phosphorus in the wash liquors. We have assumed that the amount of organic phosphorus was unaffected by washing, and the validity of this assumption is supported by the fact that the quantity of organic phosphorus in the total amount of bacteria from a definite volume of medium was proportional to the amount of growth as nearly as could be estimated, independently of the very different amount of washing they received in the different experiments. The lack of any relationship between the relative amounts of lipin/organic phosphorus ratio and the amount of washing also suggests that, as it is unlikely that they would be proportionately soluble, they must have been removed in very small amounts.

SUMMARY.

1. The organic phosphorus present in *B. coli* (grown in a synthetic media containing no organic phosphorus) consists of approximately 86% of organic phosphorus other than lipin and 14% lipin phosphorus.

2. The synthesis of the lipin phosphorus and of organic phosphorus other than lipin is discussed.

We should like to express our indebtedness to the Medical Research Council for a grant in aid of this work.

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THE RETARDATION OF LYTIC PROCESSES BY COLLOIDAL SILICA SOLUTION.

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SILICA, in colloidal solution, is known to possess curious and, at first sight, anomalous properties in relation to the lysis of red blood-corpuscles. On the one hand, Landsteiner and Jagic (1904) have shown that silica sol may act like a hæmolytic "amboceptor," so altering erythrocytes that they dissolve on the subsequent addition of fresh active rabbit serum; and, on the other, Landsteiner and Stankovic (1906) have proved that a mixture of colloidal silica and ox serum inhibits the complementary action of a hæmolytic system.

One of us (S. L. C.) showed (1922) that colloidal silica completely protects typhoid bacilli from the bactericidal action of fresh human serum, and, in attempting to explain the mechanism of this protection, carried out experiments which suggested that colloidal silica solution prevented specific hæmolysis by interfering with the action of complement, and drew the conclusion that its anti-bactericidal action is due to the same property. It seems probable that the factor at work in all these experiments is the fine precipitate produced by the addition of silica sol to serum—the "kieselsaureeiweissfällung" of Landsteiner and Stankovic.

As a preliminary to further investigations, it seemed desirable to study more closely the anti-bactericidal properties of colloidal silica solution in regard to relatively serum-soluble organisms on the one hand, and relatively serum-fast organisms on the other.

The technique adopted was that used in the earlier experiments with typhoid bacilli in 1922, and need not be described again here. It suffices to say that our more recent experiments, on precisely similar lines to those of 1922, have proved conclusively that the addition of colloidal silica solution, under the condition of our experiments, completely prevents the lysis of *B. typhosus* (Rawlings), *B. coli communis* and *B. dysenteriæ* (Flexner) by normal human blood or fresh human serum.

On the other hand, we have found that relatively serum-resistant organisms, including staphylococci, streptococci and tubercle bacilli, are just as numerous after exposure to the action of blood without admixture of silica, as after exposure to a mixture of blood and silica sol. These germs, being naturally resistant against the solvent action of blood and of blood-serum, derive no advantage from the presence of a substance capable of inhibiting "complement."

Another series of experiments with *B. typhosus*, in which the blood was left out and a similar volume of nutrient broth substituted, served to show that colloidal silica solution possesses no growth-stimulating properties *in vitro*.

These preliminary tests confirmed the conclusion already formed that the action of colloidal silica solution, in preventing the destruction of serum-soluble bacteria, depends upon an interference with the lytic action of "complement."

Earlier unpublished observations on tryptic digestion had, however, suggested that silica sol might possess anti-lytic powers apart from this interference with complement, and so we decided to examine its effects upon the lysis of pneumococcus in ox bile—a reaction in which complement is not known to play a part.

Experiment I.

A fairly strong suspension of Type I pneumococcus culture in sterile normal saline emulsion was prepared and a supply of fresh ox bile obtained. The solution of colloidal silica employed was prepared by Dr. Tytler from water-glass (Boots), according to the method described by Gye and Purdy (1922). This solution, used throughout all the following experiments, was found to give a residue of 0·878 per cent. when dried, and its activity was proved by its power to agglutinate erythrocytes in salt solution, and its antibactericidal action in relation to typhoid bacilli.

The following mixtures of the above reagents were set up in small test-tubes:

Mixture I:

Pneumococcus emulsion	5 parts.
Normal saline	4 ,,
Ox bile	1 part.

Mixture II:

Pneumococcus emulsion	5 parts.
Colloidal silica solution	4 ,,
Ox bile	1 part.

Mixture III:

Pneumococcus emulsion	5 parts.
Normal saline	5 ,,

All three mixtures were placed in the incubator at 37° C. for one hour, after which they were removed and examined.

Naked-eye appearances:

Mixture I: Clear.
Mixtures II and III: Turbid.

Hanging drop:

Mixture I: Very few pneumococci visible.
Mixtures II and III: Very many pneumococci seen.

Stained films (Gram's stain):

Mixture I: Hardly any pneumococci to be found. Much granular *débris*.
Mixtures II and III: Numerous pneumococci staining normally and showing well-marked "negative" capsules.

From this experiment it was clear that the presence of silica sol interfered to a marked extent with the lysis of pneumococci by ox bile.

This finding, which suggested that colloidal silica solution was capable of interfering with a lytic process regarded as being independent of the presence of complement, led us back to the earlier unpublished experiments on the interference of silica sol with tryptic digestion.

An attempt was now made to repeat these as follows :

Experiment II.

Allen & Hanbury's trypsin solution (" Bacteriological in Ampoules ") was used in a 1·8 per cent. solution of sodium phosphate as the digestive fluid. To this, in final concentrations of 1/20 and 1/40, slices of coagulated egg-white 3 mm. square, and cut so thin as to be almost transparent, were added, with and without 0·5 c.c. silica sol. It was found that, especially with the weaker solutions of trypsin, the digestion of egg-white was markedly retarded.

Experiment III.

This experiment was carried out with freshly prepared fibrin. About 1 c.c. of freshly drawn human blood was stirred with a thin glass rod during clotting. The fibrin, collected on the end of the glass rod, was washed in running water until nearly free from colour. Two masses of fibrin, so prepared, and of approximately equal size, were added to trypsin diluted 1/25 with phosphate, with and without an equal volume of silica sol.

The result, after two hours at 37° C., showed that whereas the control without silica was digested, with silica the fibrin was merely broken up.

These experiments indicated that tryptic digestion was interfered with to a considerable extent by silica sol, but suggested that the action was a retardation rather than an actual inhibition of lysis, the time factor being of importance. Similar conclusions were drawn from experiments in which the degree of tryptic digestion was estimated with Congo-red fibrin and also in the case of peptic digestion.

In view of the known effects of inhaled silica dust in leading to lung fibrosis and lymphatic obstruction in those exposed to it for sufficient periods of time, and in view of the theory enunciated by Gye and Kettle (1922), and now generally accepted, that " the fibrosis brought about by finely divided silica " is " due to the slow formation in the tissues of silica sol," these anti-lytic properties appertaining to or associated with colloidal silica solution appear worthy of consideration in connection with the pathology of silicosis.

All those who have examined sections from silicotic lungs are familiar with the collections of dust-cells to be seen packed tightly into, and apparently blocking up, the perivascular and peribronchial lymphatics. These cell collections may be seen months or years after the miner has ceased to be exposed to dust, and the question arises as to how the cells come to persist.

Mavrogordato (1922) suggests that "there are two reactions characteristic of free silica:

"A. That it exerts a preservative action on the cells that take it up.

"B. That cells laden with free silica tend to flock together and are preserved *en masse*, *i. e.* tend to agglutinate and form pseudo-tubercles."

To explain this preservation he suggests the following hypothesis: "That the fine particles of free silica are slightly soluble in the alkaline tissue juices and that there is a drift of alkaline silicate through the invaded cells, the principle being similar to that of the preservation of eggs by water-glass. Tissue death is associated with acid autolysis and the alkaline silicate will be precipitated as silica acid, thus, perhaps, preserving the cells as 'mummies' by impregnation."

The experiments above described appear to add significance to the suggestion of Mavrogordato as to the possibility of a prevention of acid autolysis of dust-cells by silica in solution.

As to the agglutination of these cells into masses, another curious property of colloidal silica comes to mind. Landsteiner and Jagic (1904) called attention to the agglutination of red blood-corpuscles by silica sol in the presence of salt in appropriate amount. One of us (S. L. C.) (1922, *b*) independently observed and reported the same phenomenon, and he takes this opportunity of expressing his regret that, being at that time unaware of the earlier work of Landsteiner, he failed to refer to it in his paper.

In view of these anti-lytic and cell-agglutination properties of colloidal silica, the following experiment, designed to throw further light on its effects upon a suspension of leucocytes and dust-cells, was carried out:

Experiment IV.

A heavy suspension of silica dust was made in sterile saline solution. The dust used was part of a sample given us by Dr. Mavrogordato, and represented the insoluble residue recovered from the lungs of a gold miner dying from silicosis on the Rand.

Two c.c. of this suspension were inoculated intraperitoneally into guinea-pig No. 1936 on January 6th, 1931. This animal was killed next day, and a sample of peritoneal fluid removed with sterile precautions, and mixed with an equal volume of sterile 3·8 per cent. solution of sodium citrate to prevent clotting.

Mononuclear cells larger than the usual blood monocytes were a conspicuous feature in "wet" preparations. Examined under "crossed Nichols," many of these large cells were seen to contain doubly refracting particles, presumably phagocyted silica dust.

This suspension of leucocytes and dust-cells was divided into two portions, one, labelled A, being mixed with an equal volume of sterile normal saline solution, and the other, labelled B, with an equal volume of colloidal silica solution. These mixtures were incubated in sealed capillaries, and pairs of these were examined from time to time.

The results were as follows:

Examination of Fixed Films.

8.1.31	Series A	Cells evenly distributed and well preserved.
	,, B	Cells agglutinated into irregular masses; the individual cells being well preserved and each mass apparently held together in a gelatinous matrix.
15.1.31	,, A	Cells rare and degenerated.
	,, B	Cells in agglutinated masses as before; many well preserved but some degenerated.
29.1.31	,, A	Cells very rare.
	,, B	Many cells in masses.
5.2.31	,, A	Cells degenerate and ill-stained.
	,, B	Many cells well preserved and in masses, but some are ill-stained and irregular in outline.
19.2.31	,, A	Cells all appear degenerate.
	,, B	Many cells still in masses and well preserved, though others are indistinct in outline.

Cell enumeration in Füchs and Rosenthal Cytometer.

	Series A.	Series B.
8.1.31	117 cells per c.mm.	Cells uncountable.
15.1.31	16 ,, ,,	Uncountable.
29.1.31	4 ,, ,,	29 per c.mm. ("degenerates" omitted).
5.2.31	11 ,, ,,	132 per c.mm. (estimate only).
19.2.31	13 ,, ,,	67 per c.mm.

The "counts" in Series B were always difficult owing to the large number of cells present and their agglutination in clumps. This agglutination seemed to become rather less marked from January 29th, 1931, onwards, but the counts were never satisfactory.

This experiment, by indicating that the cells were better preserved in Series B than in Series A, suggested that it was the added colloidal silica solution rather than the phagocyted silica particles which had led to the interference with the normal cytolysis observed. It seemed probable that the cells lost their vitality shortly after removal from the body, and that the tendency to slow solution of silica particles in phagocytes, postulated by various workers to occur *in vivo*, was not operative under the conditions of our experiment.

To obtain further information on this point, the following experiment was now carried out:

Experiment V.

Two guinea-pigs were inoculated intraperitoneally, one with anthracite dust in saline and the other with silica dust.

Both these animals were killed in 24 hours, and their peritoneal fluid pipetted off with sterile precautions, citrated, and the fluid from each guinea-pig put up in two series, Series A being without and Series B with added colloidal silica solution.

The results in both animals were similar to those obtained in Experiment IV, showing that it was the added colloidal silica solution which was operative in agglutinating and preserving the cells, rather than any silica which might have been dissolved from the quartz dust used to produce the cellular exudate.

It was, of course, improbable that the time allowed in these experiments would suffice to allow of the slow solution of silica particles which appears to take place in the silicotic lung. It was for this reason that we depended on the added silica sol to obtain the effects noted.

CONCLUSIONS.

1. Following upon an earlier observation that the addition of colloidal silica solution preserves typhoid bacilli from lysis in human blood or serum, we have shown that this property extends to other serum-soluble bacteria.

2. We have obtained evidence that colloidal silica solution is also able to preserve Type 1 pneumococci from lysis in ox bile.

3. Our experiments show that both tryptic and peptic digestion of albuminous substances is markedly retarded by the presence of colloidal silica solution.

4. We find that the addition of colloidal silica solution to a cellular exudate produced by the intraperitoneal injection of dust particles leads to the agglutination of the leucocytes and dust-cells and to the preservation of a large proportion of these cells, intact though probably dead, for a period of two months and upwards, beyond which time our observations have not been extended.

5. We think it probable that these anti-lytic and agglutinating properties of colloidal silica solution may, through interference with cytolysis, be factors in the production of the lymphatic obstruction associated with pulmonary silicosis in man.

We desire to express our thanks to Dr. W. H. Tytler for help and advice in the biochemical problems involved in our experiments, and the Medical Research Council for a grant which has facilitated our work.

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EFFECTS OF COLLOIDAL SILICA UPON THE GROWTH OF TUBERCLE BACILLI IN BLOOD.

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GYE and Kettle (1922, *a*) have described experiments which point to a marked adjuvant action of silica in establishing the growth of tubercle bacilli in animal tissues. These authors consider that, when tubercle bacilli are introduced into the subcutaneous tissue of mice along with silica, "a small dose of bacilli becomes a dose of considerable magnitude and, inasmuch as an important factor in determining an infectious process is the number of organisms introduced, a simple explanation is forthcoming of the effect of silica upon such tuberculous lesions as we have described."

The same authors (1922, *b*), in a second paper published in the same year, investigated the effects of silica sol on the course of a general tuberculosis in susceptible animals. They found that "silica sol circulating in the blood-stream does not materially influence a general tuberculosis. It is apparently only when the poison is sufficently concentrated upon local areas of tissue destruction that the growth of the tubercle bacillus is promoted." Kettle (1924), further to this communication, concluded that "tubercle bacilli injected into the circulation of rabbits and mice, tend to settle in localized subcutaneous lesions produced by various agents which bring about increased vascularity and tissue necrosis. . . . Inasmuch as similar results are not obtained with the provocative agents, silica appears to have some specific action on the growth of the tubercle bacillus in the tissues."

Gye and Kettle, in the light of their experiments, concluded that "the abnormal prevalence of tuberculosis among men exposed to fine silica dust may be explained by (1) the destructive action of silica on cells whereby foci of necrosis are produced in which tubercle bacilli can multiply, and (2) the disorganization of the lymphatic drainage of the lung"; and they expressed the view that "the actual cell poison is a soluble derivative of silica."

It was the enunciation of these views, in which so much stress was laid upon the importance of silica in the form of a colloidal solution, that led one of us (S. L. C., 1922) to investigate the inhibitory effects of silica sol upon the bactericidal action of human blood. It seemed likely that the presence of silica as a colloidal solution in a restricted area of tissue might favour the growth of pathogenic bacteria by preventing or diminishing the bactericidal action of the cells and the humours; and this question appeared to be capable of submission to experiment *in vitro*.

As a preliminary, it was decided to work with a germ markedly susceptible

to the bactericidal action of normal blood. An old laboratory strain of *B. typhosus* (B. T. Rawlings) was therefore used for the investigation. Experiments carried out with this organism proved that the presence of silica sol was sufficient to abolish the bactericidal action of human blood or blood-serum on *B. typhosus*. Further, it was demonstrated by hæmolytic tests on similar lines that this action of colloidal silica depended upon an interference with the lytic action of "complement."

Further experiments since performed and described elsewhere (p. 239) have shown that this antibacteriolytic action of colloidal silica is only demonstrable for serum-soluble organisms such as *B. typhosus*, *B. coli* and *B. dysenteriæ*. No such action can be demonstrated in the case of relatively serum-fast bacteria such as staphylococci and streptococci; nor is the technique employed for experiments with *B. typhosus* well calculated to bring to light any form of anti-bactericidal activity not depending on the inhibition of lytic processes.

In working with *B. typhosus* and other serum-soluble bacteria, it is necessary to restrict to 1 hour 40 minutes the time of exposure of the bacillary emulsion to the action of blood, in order to avoid the possibility of confusion of results through the rapid multiplication of a few bacteria surviving after the lysis of the majority.

In the case of a slow-growing organism such as the tubercle bacillus, however, it appeared safe to allow of a much longer time. We therefore decided to leave our cultures for a week or two instead of a few hours, and to rely, for the reading of results, not only on sub-cultures, as in the previous experiments, but on microscopic examination and the rough enumeration of tubercle bacilli or colonies in stained films.

Wright (1924) proved by special methods of culture that tubercle bacilli can grow freely in blood-clots, and at the same time afforded evidence that the aggregation of leucocytes around masses of bacilli in such preparations has a definite anti-bacterial effect. This latter point appeared of much interest in view of the suggestion by Gye and Kettle that the adjuvant action of silica upon the growth of tubercle bacilli in the tissues depends upon its being a cell-poison. Our experiments with staphylococci and streptococci had shown that there was little chance of demonstrating an anti-bacteriolytic action of silica in the protection of a germ so serum-fast as the tubercle bacillus in contact with blood; but there remained the interesting question whether the bacilli might, by this means, be protected from the anti-bacterial action of the leucocytes through the poisoning of these cells by silica.

Attempts to employ Wright's technique of growth in clots for our experiments failed, because the addition of silica sol interfered with the normal process of coagulation. It was obvious that we must devise a new technique. Here it will suffice to describe that finally selected. One of the main difficulties in work of this sort lies in the liability to air-borne contaminations. Another difficulty is to ensure sufficient aëration of the surface of the culture. This, as pointed out by Wright in his work on the growth of tubercle bacilli in clots in capillary glass tubes, is a matter of considerable importance.

For reasons already given we abandoned attempts to work with clot-cultures, and our aim was to employ instead small volumes of fluid blood.

Preparation of Tubercle Bacillary Emulsion.

From a 4-weeks culture of tubercle bacilli on egg-medium a sufficient amount of growth was removed on a platinum spatula and introduced into a thick-walled tube. Using the rounded end of a glass rod, " hooded " with a piece of cardboard to afford a shield against air contaminations, masses of tubercle culture were slowly ground up into a paste with drops of sterile 3·8 per cent. citrate solution. Finally, sufficient citrate solution was added to make a thin grey emulsion, which was then " spun " for a few minutes to remove clumps.

Preparation of Cultures.

Four 0·01 c.c. volumes of sterile blood were drawn up from the finger into a pipette, and followed by one volume of citrated tubercle bacillary emulsion, and five volumes of either sterile saline solution or of sterile colloidal silica solution.

The whole, amounting to a volume of 0·1 c.c., was now blown out into a Dreyer's tube and gently mixed by means of the pipette. The outer portion of the plug was snipped off with a sterile scissors and the remainder pushed half-way down the tube. On to this inner plug of cotton-wool a little sterile water was dropped, and the tube closed with a small rubber stopper and sealed with paraffin wax. The cultures so prepared were incubated for ten to twelve days in a sloped position.

Spreading and " Counting " of the Cultures.

The contents of each culture tube were drawn up into a sterile pipette and gently mixed on a glass slide. From this mixture a standard loopful was spread evenly over a circular area 15 mm. in diameter on a microscope slide, along with a similar loopful of a standard staphylococcus emulsion. The latter was added to provide an indication as to whether the films were evenly spread. It should be noted that the cultures containing silica tended to be rather more viscid, and to contain more *débris* than those containing saline only. All the films, made as above, were fixed by heat, stained with Ziehl and methylene-blue.

The tubercle cultures used consisted of 4-weeks-old growths on egg medium and were of origin as follows :

(1) T.B. Constantanides. Virulent strain. Isolated at Cardiff.
(2) T.B. " R. 1." From Trudeau Laboratories, Saranac (non-virulent).
(3) T.B. A. W. Jones. Strain of low virulence. Isolated at Cardiff.

The counting of the cultures was carried out as follows :

Using a $\frac{1}{6}$-in. objective and a No. 3 ocular with a square obturator, the visible " colonies " were counted in successive fields from top to bottom across the diameter of the circle. This meant that about 20 fields were counted, including the thinner central and the thicker peripheral parts of the film. By using this rather low magnification only growing " colonies " of tubercle bacilli were seen, the individual bacilli, which might have merely survived from the originally implanted emulsion, being invisible. The colonies of tubercle bacilli were found to vary much in size and consisted of wavy whorls of growth.

In order to take the size as well as the numbers of colonies into account we classified the colonies into five groups, the smallest being included in Group A, the largest in Group E, and the intermediate ones in Groups B, C and D according to size. In comparing the growths we adopted the convention of allotting 1 mark for each colony in Group A, 2 for each in Group B, and so on up to 5 marks for each colony in Group E. The total of marks for all the colonies added together was recorded as the " value " for each film. Examination of a series of fields across the same diameter with the $\frac{1}{12}$-in. oil-immersion lens and a smaller "obturator" assured us that the cocci were evenly spread, indicating that the films were roughly comparable in this respect. The results of a " count " on these lines are given in Table I:

TABLE I.—*" Colony " Counts in Blood-Cultures of Tubercle Bacilli.*

Film No.	T.B strain. Source.	Blood source.	Saline or silica.	Total colonies.	"Value" in marks.	Average number of cocci per field.
1.	Constantanides	C. W—*	Saline	59	158	47·1
2.	Ditto	C. W—	Silica	49	114	54·0
3.	R. 1	C. W—	Saline	47	129	47·3
4.	Ditto	C. W—	Silica	151	372	64·3
5.	Jones	C. W—	Saline	110	269	39·5
6.	Ditto	C. W—	Silica	78	167	30·6
7.	R. 1	K. S—†	Saline	142	353	30·9
8.	Ditto	K. S—	Silica	64	139	42·8
9.	Jones	K. S—	Saline	56	109	38·3
10.	Ditto	K. S—	Silica	5‡	5‡	32·0‡

* A healthy individual.
† An arrested case of pulmonary tuberculosis.
‡ Film 10 was not countable across the diameter of the circle as the central part washed off in staining. Only the peripheral part was visible, but this contained very few colonies.

These " counts " show that all the cultures contained growing colonies of tubercle bacilli. The numbers of colonies and the " colony value " was greater in the case of the " saline " cultures than in the " silica " cultures except in Film 4, which, as indicated by the staphylococcus count, was an unusually thick film. The differences, however, are not sufficient to be significant, and it is clear, from this and other similar experiments, that the growth of tubercle bacilli in blood *in vitro* is neither favoured nor inhibited by the presence of colloidal silica solution.

From a similar series of preparations grown at 37° C. for 12 days as above, cultures on egg medium were made and incubated. The growths, after five weeks' incubation of the egg media, were well marked in all, being, however, rather more profuse in those tubes inoculated from the " blood-saline " than in those from the " blood-silica " preparations.

It is interesting to note that, in the blood of K. S—, an " arrested " case of pulmonary tuberculosis, there appeared to be more profuse growth of tubercle bacilli in the " saline " than in the " silica " mixtures. Had the silica possessed any considerable anti-bactericidal power, it might have been expected to show

it here by affording a greater protection, and leading to a more marked growth of tubercle bacilli in a blood presumably fairly active against the tubercle bacillus.

Our experiments "*in vitro*," all being variations of the general scheme of investigation above described, lead us to the conclusion that silica sol does not in any way further the pullulation of tubercle bacilli in human blood outside the body.

These experiments have been facilitated by a grant from the Medical Research Council, for which we desire to express our thanks.

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THE INHIBITORY ACTION OF SANOCRYSIN ON TUBERCULOUS LESIONS.

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EVER since the introduction of sanocrysin as a therapeutic agent for the treatment of tuberculosis, attempts to establish its value experimentally in animals have been very conflicting. Cummins (1926) obtained successful results in rabbits, but Bang (1926) could find no curative action whatever in calves, rabbits or guinea-pigs. Madsen and Mörch (1926) published experiments showing complete sterilization of rabbits injected with the same strain of bovine tubercle bacillus as Bang employed in his work cited above, but when later they came to repeat the work in a parallel series with Bang (1928) they could not show any effect of sanocrysin on the disease. In this state of confusion it occurred to the author that some light might be thrown on the obscure subject of the mode of action of sanocrysin by testing its effect, if any, on tuberculous lesions, caused by dead tubercle bacilli. It is well known that dead bacilli produce tuberculous lesions of a similar character to those caused by the living organism when introduced into an animal. In this way the complicating factor of the living bacillus reproducing itself and causing further lesions is entirely eliminated ; the extent of the lesions is more under control. The trend of opinion among pathologists at the present time is in favour of

the view that chemo-therapeutic substances act by co-operating with the tissues of the host in resisting invasion rather than by exerting a bactericidal action. There is reason to suppose, therefore, that sanocrysin might modify in some way the lesions caused by the tubercle bacillus, quite apart from any bactericidal effect it might have on that organism itself.

The principle adopted in the present work has been to inoculate rabbits with a dose of killed tubercle bacilli capable of producing lesions attended by allergy of such intensity that the animals succumb to an intravenous injection of "old tuberculin." The power of sanocrysin to influence this sequence of events has been tested.

EXPERIMENTAL.

The tubercle bacillus used was an old bovine strain isolated in 1923, now of low virulence for rabbits. It was grown on Petroff's medium for about a fortnight, the growth scraped off and emulsified in physiological salt solution. It was killed by heating in a water-bath at 60° C. for one hour. It had previously been found that this treatment was more than sufficient to ensure the death of the bacilli.

Experiment 1.

In the first experiment with sanocrysin the bacillus was grown on Petroff's medium for 14 days. It was then scraped off, weighed and smeared on the walls of a test-tube with a glass rod. By adding salt solution drop by drop a good homogeneous emulsion was obtained. It was heated, during which process a considerable degree of flocculation occurred, but the masses could readily be dispersed by drawing through a capillary glass pipette held against the bottom of the tube just before injection. The rabbits were all young, the weights varying from about 1200–1500 gm. The dose of sanocrysin given was 0·02 gm. per kg. body-weight, as Madsen and Mörch (1928) proved this to be nearly the maximum amount which a rabbit would tolerate, for when twice the dose was given about half the animals died after a couple of injections from metal poisoning.

An autopsy was made on the rabbits that succumbed to the tuberculin injection. The lungs were usually very red, the intensity of the inflammation varying in the two lungs and in different parts of the same lung. The spleen was congested, but the liver, to the naked eye, appeared normal. Sections were cut of the liver and lungs. The rabbits that survived the tuberculin injection and seemed to have recovered from any malaise they may have had from a sub-lethal effect, were killed after the expiration of 48 hours, and pieces of the liver and lungs taken for section. The microscopic appearance of the lesions in the liver was very characteristic. Early tubercle in the form of epithelioid cell aggregates occurred round both interlobular and intralobular vessels. Giant-cells were usually present also, although their numbers were very variable. They occurred for the most part outside the areas of epithelioid cells. The extent of the tuberculous lesions corresponded with the result of the tuberculin injections. When large in amount—the foci of tubercle originating round a vessel having grown to a large size and often merged with

neighbouring ones—it was practically always found that the rabbit was killed by the intravenous tuberculin injection. Indeed this was so constant that from an examination of a section of the liver alone a good guess could be made as to whether the rabbit died from the tuberculin or not. When the animal survived, the lesions were much less in size, sometimes being restricted to a minimum amount of tuberculous infiltration around some of the vessels. This means, therefore, that the degree of allergy developed is roughly proportional to the amount of tubercle present.

TABLE I.—*Action of Sanocrysin on Lesions caused by Dead Tubercle Bacilli.*

Day of experiment.	Rabbits' Nos.	Injection.
1	1–16	0·01 gm. dead tubercle bacilli intravenously.
	1, 3, 5, 7, 9, 11, 13, 15	0·02 gm. sanocrysin per kg. weight shortly before the injection of tubercle bacilli.
3	,,	0·02 gm. sanocrysin per kg.
5	,,	,, ,, ,,
8	,,	,, ,, ,,
10	,,	,, ,, ,,
12	,,	,, ,, ,,
15	,,	,, ,, ,,
17	,,	,, ,, ,,
19	,,	,, ,, ,,
22	,,	,, ,, ,,
24	,,	,, ,, ,,
26	,,	,, ,, ,,
30	1–14 and 16*	1 c.c. old bovine tuberculin intravenously.

Result: Nos. 1, 2, 3, 4, 6, 10, 12, 14 and 16 died within 24 hours.

* No. 15 was found dead on the 23rd day.

Experiment 2.

In the second experiment the same procedure in making the emulsion of tubercle bacilli was adopted except that one-fifteenth molecular secondary sodium phosphate (Na_2HPO_4) was used instead of physiological salt solution. The change was made owing to the difficulty experienced in preparing another such good emulsion of the bacilli as was used in the first experiment. The solution is alkaline and there is less tendency to spontaneous clumping than with ordinary salt solution. As a further precaution the emulsion, after the heating and thorough breaking up of the clumps of bacilli which always form in the process, was allowed to stand so that the largest of the remaining clumps could settle to the bottom of the tube and so be removed. In this way a comparatively good emulsion was obtained. The great difficulty in such work as this

is to prepare a suspension of tubercle bacilli sufficiently free from large clumps, which, if present, vitiate the experiment by lodging in the lungs of some of the rabbits, and causing rapid death from the production of a relatively large amount of local tubercle in that organ.

As the quantity of tubercle in the present emulsion was uncertain on account of the removal of the deposited clumps, a preliminary test was made on a series of rabbits to find the dose which would render them so allergic in about a month that they would die from a tuberculin injection. The dose was found to be roughly 0·75 c.c. of the emulsion. On the 23rd day of the experiment, it was decided to give the tuberculin injection without delay as the controls appeared to be affected, judging from their general condition.

TABLE II.—*Action of Sanocrysin on Lesions caused by Dead Tubercle Bacilli.*

Day of experiment.	Rabbits' Nos.	Injection.
1	1–12	0·75 c.c. emulsion of dead tubercle bacilli intravenously.
	1, 2, 3, 4, 5, 6	0·02 gm. sanocrysin per kg. weight shortly before the tubercle bacilli injection.
3	1–6	0·02 gm. sanocrysin per kg.
6	,,	,, ,, ,,
8	,,	,, ,, ,,
10	,,	,, ,, ,,
13	,,	,, ,, ,,
16	,,	,, ,, ,,
18	,,	,, ,, ,,
20	,,	,, ,, ,,
23	1–6 and 8–12*	1 c.c. old bovine tuberculin intravenously.

Result: Nos. 8, 10, 11 and 12 died within 24 hours.

* No. 7 died in the morning before the tuberculin injections were given.

As in the previous experiment the amount of tubercle found in the liver was proportional to the degree of allergy developed. The same also applies to the tubercle in the lungs.

Results of the Experiments.

In the first experiment the tuberculin injection proved fatal in two rabbits treated with sanocrysin and 7 untreated controls; while 5 rabbits treated with sanocrysin and only 1 control survived. (One of the sanocrysin-treated rabbits died before the tuberculin test was done, with fairly advanced tubercle in the lungs—caseating areas also being present.) *In other words* 5 *out of* 7 (8) *rabbits were protected by sanocrysin from a lethal effect of a tuberculin injection, but only* 1 *out of* 8 *of the untreated controls survived the test.* The two rabbits,

Nos. 1 and 3, which were the only ones treated with sanocrysin to die as a result of the tuberculin, weighed about 1200 gm., while some of the others weighed nearly 1500 gm. Consequently they received a rather larger dose of tubercle bacilli compared with their weight. The tuberculous lesions found in the liver were in conformity with the above findings in that those rabbits which were killed by the tuberculin had the most extensive tuberculous lesions.

In the second experiment all the 6 rabbits treated with sanocrysin survived the tuberculin injection and had comparatively small histological tuberculous lesions in the liver ; but 4 of the 5* controls which had had no sanocrysin died within 24 hours with relatively large areas of tuberculous infiltration in the liver. This experiment demonstrates the protective action of sanocrysin even more strikingly than the first did.

DISCUSSION.

The conclusion to be drawn from these experiments is that *sanocrysin tends in some way to prevent the formation of tubercle.* A very definite inhibitory effect has been demonstrated on both the development of allergy and the growth of tuberculous lesions, the two things being intimately related, if, indeed, there is not a direct causal connection between them. That the protection is only partial under the conditions of the experiment is shown by other experiments of a similar kind in which the tuberculin injection was delayed, when as many sanocrysin-treated rabbits as controls died after it, and the histological lesions had advanced to the same stage in both. It seems possible, therefore, that if the experiments recorded above had been continued for a considerably longer period, the tuberculous lesions in the sanocrysin-treated rabbits might have gradually reached the proportions of those in the controls, and have been attended by sufficient allergy to cause death from a tuberculin injection. But the interesting point is that an inhibitory action of sanocrysin on the development of tubercle has been shown to exist which is distinct from any possible lethal effect it also may have on the tubercle bacillus. This, however, is not to say that it does not act on the bacillus or its toxic substances. The explanation of the result obtained must be either such a destructive action on the irritative bodies provided by the bacillus, or some obscure interaction with the tissues resisting the formation of tuberculous lesions. That it destroys these lesions after they have come into existence is unlikely, for there is no histological evidence for such a view, and the time available is extremely short. Nor can the sanocrysin merely have depressed the state of allergy, because the tuberculous lesions were at the same time very restricted in extent, the allergy developed apparently depending on the size of these lesions.

It is worth pointing out that experiments such as these afford a ready means for testing the efficacy of new therapeutic remedies in tuberculosis. They are of relatively short duration, lasting only a month, and could be used for eliminating probably inactive drugs before proceeding to the more complicated and difficult work of testing their power in the presence of living tubercle bacilli, with which naturally the final decision must rest. Further, the size

* One having died before the time for the tuberculin injection.

and spacing of doses of the drug to ensure the optimum effect might be more easily settled in this way.

CONCLUSIONS.

1. Rabbits injected intravenously with heat-killed bovine tubercle bacilli were protected by repeated injections of sanocrysin from the lethal action of an intravenous injection of old tuberculin about a month later, while control animals that had received no sanocrysin succumbed. In conformity with this the controls showed extensive histological tuberculous lesions in the liver, but those rabbits treated with sanocrysin had slight, or at any rate, less extensive ones corresponding with the considerably smaller degree of allergy developed.

2. The experiments prove that *sanocrysin exerts an inhibitory effect on tuberculous lesions, apart from any bactericidal action it may have on the living tubercle bacillus.*

3. It is pointed out that such experiments form a ready means for testing the possible efficacy of new therapeutic drugs in tuberculosis and the best methods of administering them.

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IMMUNIZATION WITH KILLED HERPES VIRUS.

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UNTIL comparatively recently it was generally accepted that filterable viruses gave rise to little or no immune response unless they were employed in the living state; rabies virus provides an exception to this statement. The demonstration, however, that the viruses of foot and mouth disease (Vallée, Carré, Rinjard, 1925; Bedson, Maitland and Burbury, 1927), distemper (Laidlaw and Dunkin, 1928) and yellow fever (Hindle, 1928) can be inactivated by low concentrations of formalin, yet retain their antigenic power to a considerable degree has materially altered the outlook, and at the present time it is admitted that virus rendered inactive by such means is still capable of producing an appreciable degree of immunity. But the question as to whether dead virus can immunize is still in dispute, many workers holding that these inactive vaccines contain a residuum of living virus, and that their efficacy is

attributable to this living residue. The point is one of considerable interest and of practical importance, for if inactive vaccines of filterable viruses are to be used in prophylaxis—and there is reason to think they will be of service, particularly if the problem of cultivation of filterable viruses on artificial media is solved—their usefulness would be curtailed considerably if their efficacy depended on a variable quantity of living virus. It was in order to find out whether herpes virus inactivated by formalin possessed any immunizing power, and if so whether this was due to dead virus, that the following experiments with herpes virus were carried out.

EXPERIMENTAL.

Herpes virus adapted to the guinea-pig's skin was used in this work. Primary inoculation from human herpes vesicles was made in the skin of the guinea-pig's foot, and the strain maintained by weekly passage in the same tissue. Two strains were used, both of which were well adapted to the guinea-pig, and which maintained an almost constant titre. A skin strain of herpes virus was chosen in preference to one adapted to the brain or testicle, because the availability of skin titration made possible the quantitative estimation of immunity with a smaller expenditure of animals.

Choice of Experimental Animal.

The guinea-pig was selected in preference to the rabbit because it is more readily immunized; to this extent the experiments were biased in favour of the vaccines. There is a tendency to regard the guinea-pig as relatively insusceptible to herpes virus, but nothing could be more incorrect. During the last few years the author has isolated a considerable number of herpes strains by direct inoculation from man to guinea-pigs, and although a few of those strains lapsed by the fourth of fifth passage, the majority were readily maintained in the guinea-pig in an active state. The guinea-pig, however, is less susceptible to herpes virus than the rabbit.

Criterion of Death of Virus.

It is quite certain that simple inoculation is insufficient to determine whether all the virus in a given suspension is dead; a negative result would not rule out a sub-infecting dose of living virus. It has been found possible to reactivate sub-infective doses of herpes virus by serial passage in the plantar skin of the guinea-pig, so this procedure was made use of to determine whether any living virus remained in the formalized vaccine. If serial passage through three guinea-pigs failed to reveal living virus, the vaccine was considered to be composed entirely of dead virus.

Immunization with Formalized Virus.

Preparation of Vaccine (*B.*893/6).

Herpetic guinea-pig pads weighing 2·4 gm. were ground with sand and 4·8 c.c. 9 per cent. salt solution added. After standing overnight in the refrigerator, sterile distilled water was added to bring the volume to 48·0 c.c., the

whole thoroughly mixed, centrifuged lightly, and the supernatant pipetted off. The suspension was then titrated for virus, the 10^{-3} dilution proving infective, while 10^{-4} was negative. Formalin (40 per cent. formaldehyde) was added in a concentration of 0·2 per cent., and the suspension placed in the refrigerator. After ten days in the refrigerator the vaccine was tested for the presence of active virus by serial passage through three guinea-pigs. This occupied twelve days and proved negative.

Efficacy of Vaccine.

Immunization of guinea-pigs was commenced when the vaccine B.893/6 was 22 days old. The details of immunization and the resulting immunity are to be found in Table I, and require no further description. The neutralizing power of the serum of the immunized guinea-pigs was tested seven days after the last dose of vaccine; the details of these estimations are given in Table II. The experiment shows that this vaccine, which had been formalized for twenty-two days prior to use, and contained less active virus than could be reactivated by serial passage through three guinea-pigs, was capable of exciting a high degree of immunity in guinea-pigs.

TABLE I.—*Immunization with Formalized Herpes Virus.*

Guinea-pig.	Immunization.	Weight in gm. Before.	Weight in gm. After.	Test of immunity. Intradermal inoculation of falling concentrations of virus in 0·2 c.c. volume. Test made 10 days after last dose of vaccine. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
B.914	Three doses of 1·0 c.c. of vaccine B.893/6 given subcutaneously at intervals of 7 days	495	535	A	—	—	—
B.915		580	720	A	—	—	—
B.916		538	580	A	—	—	—
B.917		500	560	A	—	—	—
B.936	None	..	..	++	+	±	—

A. = Transitory allergic reaction. ++ to ± = degrees of reaction to virus.

TABLE II.—*Neutralizing Power of Serum of Guinea-pigs Immunized with Formalized Herpes Virus.*

Serum.	Final concentration of serum. 1 in 2.	1 in 10.	1 in 25.	1 in 50.
B.914	—	∓	±	+
B.915	—	—	—	—
B.916	—	—	—	+
B.917	—	—	—	+
Normal control	++	++	++	..

Equal parts of herpes virus diluted 1 in 20 in M/50 phosphate pH 7·6 and serum dilutions in phosphate. Allowed to stand for 1 hour at room temperature. Intradermal inoculation of 0·2 c.c. quantities of the various mixtures. Titre of virus used in test, 1 in 1000.

Immunization with Heat-killed Virus.

This experiment was carried out in order to see whether herpes virus killed by heat was capable of producing an immunity comparable with that given by formalized virus.

Preparation of Vaccine (*B.*982).

Herpetic guinea-pig pads weighing 0·8 gm. were ground with sterile sand, and 1·6 c.c. 9 per cent. salt solution added. After standing overnight in the refrigerator sterile distilled water was added to bring the volume up to 16·0 c.c., the whole thoroughly mixed, lightly centrifuged, and the supernatant pipetted off. The virus titre of this suspension was 10^{-3}. It was inactivated by heating for 45 minutes at 60° C. The presence of active virus was tested for in the way described with a negative result.

*Efficacy of Vaccine B.*982.

The findings in this experiment are given in detail in Table III, and leave no doubt that herpes virus inactivated by heat has little or no immunizing power.

TABLE III.—*Immunization with Herpes Virus Killed by Heat* (60° *C. for* 45 *Minutes*).

Guinea-pig.	Immunization.	Weight in gm. Before.	Weight in gm. After.	Test of immunity. Intradermal inoculation of falling concentrations of virus in 0·2 c.c. volume. Test made 12 days after last dose of vaccine. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
B.987	Three doses of 1·0 c.c. of vaccine B.982 given subcutaneously at intervals of 5 days	440	410	++	+	±	∓
B.988		440	440	+	±	∓	−
B.989		560	490	+	±	∓	−
B.990	None	..	..	++	+	±	−

Immunizing Power of Formalized Normal Guinea-pig-Pad Tissue.

The possibility existed that the immunity produced by the formalized vaccine was due to the formalized guinea-pig tissue, and not to the virus which it contained. The preparation of this control vaccine was similar to that of vaccine B.893/6, with the exception that normal guinea-pig pads replaced herpetic ones. The negative results obtained with this control vaccine given in Table IV show that the efficacy of vaccine B.893/6 must have depended on the virus present in it.

TABLE IV.—*Immunization with a Formalized Suspension of Normal Guinea-pig-" Pad " Tissue.*

Guinea-pig.	Immunization.	Weight in gm. Before.	Weight in gm. After.	Test of immunity. Intradermal inoculation of falling concentrations of virus in 0·2 c.c. volume. Test made 11 days after last dose of vaccine. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
B.979	Three doses of 0·1 c.c. of vaccine given subcutaneously at intervals of 5 days	420	400	++	+	±	−
B.980		420	430	++	+	±	−
B.1003	None	..	..	++	+	±	−

Comparison of Immunity Produced by Equal Quantities of Living and Formalized Herpes Virus.

This was compared by giving three guinea-pigs one inoculation each of the formalized vaccine B.893/6, and three other guinea-pigs an equivalent amount of living virus. The neutralizing power of the serum of these animals was tested before and 8 days after immunization. The results of this experiment are given in Tables V and VI. As one would expect, the living virus gave the better immunity, but the one dose of formalized vaccine gave quite a surprising degree of immunity—in two out of the three animals practically as much as that evoked by the living vaccine. This experiment of itself rather negatives the viewt hat the activity of the formalized vaccine was due to a residue of living virus. This point, however, is dealt with specifically in the next experiment.

TABLE V.—*Comparison of Immunity Produced by Equal Quantities of Living and Formalized Herpes Virus.*

Guinea-pig.	Immunization.	Weight in gm. Before.	Weight in gm. After.	Test of immunity. Intradermal inoculation of falling concentrations of virus in 0·2 c.c. volume. Test made 7 days after last dose of vaccine. 10^{-1}	10^{-2}.	10^{-3}.	10^{-4}.
B.952	One dose of 1·0 c.c. living herpes virus subcutaneously. (Contained the same amount of virus as the vaccine before formalization)	440	385	±	∓	−	−
B.953		445	420	+	±	−	−
B.954		425	410	+	±	?	−
B.955	One dose of 1·0 c.c. of formalized vaccine B.893/6 given subcutaneously.	440	380	++	+	+	±
B.956		420	380	±	∓	−	−
B.957		380	360	+	∓	−	−
B.972	None	..	..	++	+	±	∓

TABLE VI.—*Neutralizing Power of Serum of Guinea-pigs Immunized with Single Doses of Living and Formalized Herpes Virus.*

Serum.	Immunization.	Final concentration of serum. Before Immunization. 1 in 2.	After immunization. 1 in 2.	1 in 10.	1 in 20.	1 in 50.
B.952	One dose of living virus	+	−	−	−	±
B.953		+	−	−	∓	±
B.954		+	−	−	±	+
B.955	One dose of formalized virus	+	−	?	±	+
B.956		+	−	±	+	+
B.957		+	−	−	+	+

Equal parts of herpes virus diluted 1 in 100 (titre 1 in 1000), and serum dilutions in M/50 phosphate pH 7·6. Inoculated intradermally in 0·2 c.c. quantities after 1 hour at room temperature.

Immunizing Power of Minimal Doses of Living Herpes Virus.

Preparation of Vaccine (R.25.)

A virus suspension (guinea-pig pad) whose titre was 10^{-3} was diluted a thousandfold with a suspension of normal guinea-pig-pad tissue. Since a

dilution of 10^{-4} of this virus suspension produced no lesion on primary inoculation, but could be shown to contain active virus by serial passage, this vaccine contained ten times more virus per unit volume than could be demonstrated by this procedure, and must therefore have contained considerably more than the hypothetical quantity of living virus remaining in the formalized vaccine.

Efficacy of Vaccine.

Four guinea-pigs were immunized, the dosage and route of inoculation being the same as in the case of the formalized vaccine. The results are given in Table VII, and leave no doubt that such small doses of living virus are devoid of immunizing power.

TABLE VII.—*Immunization with Minimal Doses of Living Herpes Virus.*

Guinea-pig.	Immunization.	Weight in gm. Before.	Weight in gm. After.	Test of immunity. Falling dilutions of herpes virus inoculated by scarification. Test made 9 days after last dose. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
B.1136	Three inoculations of 1·0 c.c. subcutaneously of vaccine R.25. Each dose of vaccine contained living virus diluted 1 in 1000 (titre of virus, 1 in 1000)	700	745	++	+	?	−
B.1137		520	590	++	+	?	−
B.1138		600	615	++	+	−	−
B.1139		490	500	+	±	−	−
B.1141	None	..	..	++	+	−	−

Effect of Heating at 100° C. on Formalized Virus.

It might be argued that treatment of herpes virus with low concentrations of formalin attenuated the virus but did not kill it. Such an attenuated virus might be capable of multiplication in the animal's tissues and yet not produce an herpetic lesion. A virus suspension B.1088/9 was prepared in the way described; its titre was 1 in 1000. This was divided into two portions, one of which was steamed for twenty minutes, whilst to the other formalin was added to a concentration of 0·1 per cent. Both were stored in the refrigerator. After 7 days' formalization this second portion was divided in two, one of which was steamed for twenty minutes. These three vaccines, steamed, formalized and steamed and formalized, were then used to immunize 6 guinea-pigs, 2 with each vaccine. The resultant immunity is shown in Table VIII, and the neutralizing power of the serum of these animals 7 days after the last inoculation is given in Table IX. A duplicate experiment gave a very similar result, and the fact that herpes virus which has been formalized and then steamed retains some antigenic power, though not as much as after formalization alone, whereas the steamed virus is devoid of immunizing power, suggests that formalin does not act by attenuating the virus, but that it kills it without destroying the majority of its antigenic power.

TABLE VIII.—*Immunization with Herpes Virus Inactivated by:* (A) *Formalin,* (B) *Formalin followed by Steaming,* (C) *Steaming.*

Guinea-pig.	Immunization.	Weight in gm. Before.	Weight in gm. After.	Test of immunity. Intradermal inoculation of falling concentrations of virus in 0·2 c.c. volume 10 days after last dose. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
B.1094	Received 3 doses of 1·0 c.c. steamed virus subcutaneously at 5 days' interval	400	450	+	+	±	∓
B.1095		390	420	+	±	∓	−
B.1096	Received 3 doses of 1·0 c.c. formalized and steamed virus subcutaneously at 5 days' interval.	435	370	+	±	−	−
B.1097		350	370	±	∓	−	−
B.1098	Received 3 doses of 1·0 c.c. formalized virus subcutaneously at 5 days' interval	380	400	−	−	−	−
B.1099		380	370	−	−	−	−
B.1107	None	..	..	++	+	±	−

TABLE IX.—*Neutralizing Power of Serum of Guinea-pigs B.*1093/1099.

Serum.	Immunization.	Final concentrations of serum. 1 in 2.	1 in 10.	1 in 20.
B.1094	Steamed virus	±	+	+
B.1095		±	+	++
B.1096	Formalized and steamed virus	−	∓	±
B.1097		−	∓	±
B.1098	Formalized virus	−	−	−
B.1099		−	−	−

CONCLUSIONS.

1. Herpes virus treated with low concentrations of formalin is capable of producing a high degree of immunity in guinea-pigs.
2. This immunity is produced by dead virus.
3. Herpes virus killed by heating at 60° C. or 100° C. is devoid of immunizing power.
4. If the virus is treated with formalin before heating it at 100° C. it is still capable of producing some immunity.

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STUDIES ON IMMUNITY TO HÆMOLYTIC STREPTOCOCCI. III.—OBSERVATIONS ON THE VARIATIONS IN RESISTANCE TO PHAGOCYTOSIS DISPLAYED BY BROTH CULTURES OF STRAINS OF HIGH AND LOW VIRULENCE.

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In a previous paper (Hare, 1929) it was shown that during the incubation of recently isolated hæmolytic streptococci in broth or in human serum at 37° C., the resistance to phagocytosis by the polymorphonuclear leucocytes of normal human blood varies considerably; being most marked during the period of active growth, and gradually disappearing as the culture grows older. There is every probability that the same cycle of changes occurs when streptococci reach the serum in which a wound, or the interior of a recently delivered uterus, is bathed. And in view of this it became of importance to investigate whether the amplitude of this cycle varies in any way with alterations in the virulence of the strain. This forms the subject of this communication. It will be shown that strains which are highly virulent for mice become very much more resistant to phagocytosis and retain that resistance very much longer during incubation in broth than other strains which are less virulent; while avirulent strains do not become resistant at any period of their growth-cycle. In addition, observations which have been made on the changes in the cocci which render them more or less susceptible to phagocytosis will also be reported.

EXPERIMENTAL PROCEDURES.

Method of testing the variations in resistance to phagocytosis.—A 3-hour culture in normal horse-serum of the strain employed was kept at 0° C. during the period of each experiment. 50 c.mm. of this culture was added to a fresh tube of 5 c.c. of 5 per cent. horse-serum broth at definite intervals. These tubes were incubated at 37° C. until required. When broth cultures of a sufficiently wide variety of ages had been collected by these means, they were all tested together, employing one specimen of normal human blood. The actual technique employed for these tests has been described before (Hare, 1929), but consists essentially in:

(*a*) Each culture is concentrated or diluted to bring the total number of organisms present to about 100,000 per 1 c.mm.

(*b*) The number of cocci in the suspension made from each culture is estimated by means of a vaccine count. (Method of Wright.)

* Working with a grant from the Medical Research Council.

(*c*) One part of the microbic suspension and nine parts of normal human defibrinated blood are incubated on a mixing machine (Todd, 1927) for 15 minutes, and a film of the mixture is made with an opsonic spreader.

(*d*) The number of cocci in 100 polymorphonuclear leucocytes is counted.

(*e*) The total number of white cells and the percentage of these which are polymorphonuclears are then ascertained.

(*f*) From these figures, the total number of cocci taken up by the polymorphonuclears of 1 c.mm. of the blood can then be calculated.

(*g*) Knowing the number of cocci added (from (*b*)) it is then an easy matter to calculate the percentage of these which are taken up in the 15 minutes mixing.

These estimations having been carried out with each culture, it is possible to judge the variations in amenability to phagocytosis on the part of the cocci after varying periods of incubation in the broth.

Construction of the curve of growth.—Five c.mm. of a suitable dilution in broth of each culture were explanted into melted blood agar in petri dishes. The number of colonies enumerated after incubation (counting colonies as units) provides the necessary data for the construction of curves to show the variation in number of living cocci in the cultures from time to time.

Virulence tests with mice.—These were carried out with 16-hour 5 per cent. serum broth cultures of the strains under examination. 0·5 c.c. quantities of suitable dilutions in plain broth were injected intraperitoneally into mice without delay. The blood of the heart from all those dying was cultured in broth to ascertain the presence of hæmolytic streptococci.

Source of strains.—Hæmolytic streptococci isolated from the blood or some septic focus of cases of puerperal fever were employed. All gave good areas of hæmolysis on human or horse blood agar plates. Such strains were rendered virulent by mouse passage and avirulent by continued subcultivation on plain agar.

Note.—It is necessary to point out that phagocytosis by the polymorphonuclears is alone considered in this work. The large mononuclear leucocytes do take part in the phagocytosis of streptococci, but the total amount, in comparison with that effected by the polymorphs, is small. Phagocytosis by these cells has, therefore, been neglected.

The period of mixing is always 15 minutes. This is quite sufficiently long to secure complete phagocytosis of a non-resistant culture (Hare, 1928). Longer periods of mixing do not lead to any greater amounts of phagocytosis.

THE EFFECT OF PREVIOUS TREATMENT UNDERGONE BY THE STRAINS ON THE CYCLE OF RESISTANCE TO PHAGOCYTOSIS DURING GROWTH IN BROTH.

In the first experiment, virulent and avirulent variants of a strain C are incubated in serum broth, the curve of growth constructed, and the resistance of the cocci to phagocytosis measured from time to time. This is intended to show, firstly, that the virulent strain becomes resistant to phagocytosis during

the phase of active growth, while the avirulent does not; secondly, that the avirulent strain does not become resistant at any period of the growth-cycle; and thirdly, that although the resistance to phagocytosis of the virulent strain persists over a prolonged period of the phase of decline, it does, eventually, disappear.

Experiment 1.

Preparation of strains.—Original strain C, derived from the blood of a case of septicæmia following abortion. Two variants had been prepared:

(*a*) Had received 3 passages through mice.

(*b*) Had received 11 subcultures on agar.

Resistance to phagocytosis studied by the method described (Hare, 1929). Defibrinated blood from R.H. was used in both estimations.

That employed for (*a*) had a white cell-count of 6600 cells per 1 c.mm.; 60 per cent. were polymorphonuclears. (*b*) had a white cell-count of 6384 cells per 1 c.mm.; 60 per cent. were polymorphonuclears.

Virulence for mice studied as described.

Results are given in Tables I and II and in Chart 1.

TABLE I.

Virulent Strain.

Time of incubation of the broth culture.	Number of cocci taken up by 100 polymorphs.	Total number taken up in 1 c.mm. of blood.*	Total number present in 1 c.mm. of the blood.†	Percentage of cocci phagocyted.
8 hours	2	72	12,000	0
12 ,,	4	144	16,000	0
16 ,,	13	468	16,700	2
24 ,,	13	468	8,800	5
35 ,,	84	3,024	11,800	25
71 ,,	108	3,888	12,700	30
136 ,,	245	8,820	28,000	31
160 ,,	449	16,164	17,000	95

Avirulent Strain.

8 hours	814	27,676	29,000	95
12 ,,	408	13,872	22,300	64
16 ,,	223	7,582	12,000	63
24 ,,	162	5,508	8,400	65
40 ,,	246	8,366	11,800	71
64 ,,	278	9,452	17,500	58
84 ,,	207	7,038	8,590	82
136 ,,	198	6,732	12,500	54

† Calculated from a vaccine count made by the method of Wright.

* Calculated from the figures in column 2.

TABLE II.

Virulent Strain.

No. of mouse.	Number of organisms injected.	Day of death after injection.	Hæmolytic streptococci in heart's blood.
1	0·7	2 days	Present.
2	7	Survived 34 days	..
3	72	2	Present.
4	720	2	,,
5	7,200	2	,,
6	72,000	2	,,

Avirulent Strain.

1	1·6	Survived 24 days	..
2	16	,, ,,	..
3	160	,, ,,	..
4	1,600	,, ,,	..
5	16,000	,, ,,	..
6	160,000	,, ,,	..

Comments on this Experiment.

(1) The virulent strain is highly resistant during the logarithmic phase of growth. This resistance is still marked when the organisms are far into the phase of decline, but eventually disappears altogether.

(2) The avirulent strain is resistant at no period of its growth-cycle.

In Chart 2 is given the result of an exactly similar experiment with two variants of another strain A. In this instance, however, the less virulent of the two strains does become somewhat resistant during the logarithmic phase of growth. And the mouse tests indicate that the strain possesses some virulence.

It may be concluded from these two experiments and a number of others giving similar results, that it is an attribute of virulent strains of hæmolytic streptococci to become resistant to phagocytosis during the phase of active growth. During the stationary period and the decline phase of the culture, this resistance gradually disappears. But it only does this slowly, so that the culture has almost sterilized itself before all the organisms can be taken up by the leucocytes. Avirulent strains, on the other hand, are not resistant at any time, even during the phase of active growth, while strains of intermediate virulence occupy an intermediate position, becoming somewhat resistant during the logarithmic phase and losing resistance relatively early.

It is thus evidently one of the attributes of virulence that the cocci are not only resistant during their phase of active growth, but are resistant for quite a long period thereafter. Less virulent strains are both less resistant during the phase of growth, and also part with this resistance very much sooner.

Question as to whether the Cocci still Alive in Old Broth Cultures are Actually Losing Resistance to Phagocytosis.

It must be pointed out that in the process of estimating the resistance to phagocytosis of old broth cultures of hæmolytic streptococci, the cocci which

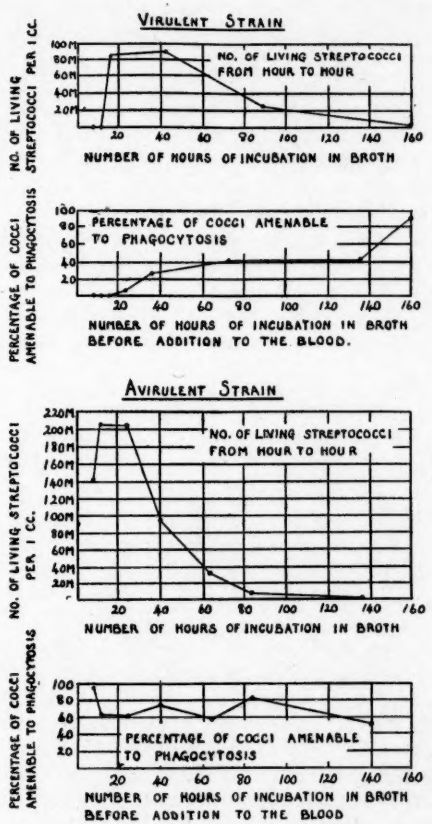
VIRULENT STRAIN
NO. OF LIVING STREPTOCOCCI PER 1 CC.
NO. OF LIVING STREPTOCOCCI FROM HOUR TO HOUR
NUMBER OF HOURS OF INCUBATION IN BROTH
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
NUMBER OF HOURS OF INCUBATION IN BROTH BEFORE ADDITION TO THE BLOOD.
AVIRULENT STRAIN
NO. OF LIVING STREPTOCOCCI PER 1 CC.
NO. OF LIVING STREPTOCOCCI FROM HOUR TO HOUR
NUMBER OF HOURS OF INCUBATION IN BROTH
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
NUMBER OF HOURS OF INCUBATION IN BROTH BEFORE ADDITION TO THE BLOOD

CHART 1. TO SHOW VARIATION IN RESISTANCE TO PHAGOCYTOSIS DURING GROWTH OF VIRULENT AND AVIRULENT VARIANTS OF STRAIN C. IN BROTH

are counted include both living and dead specimens, with, in such old cultures, the latter in the vast majority. That these can be taken up by the leucocytes is hardly surprising. Those still living may, however, be resistant, but the method employed is too coarse to permit of the detection of this. It must

therefore be admitted that the condition as regards their resistance to phagocytosis, of the still living cocci in an old broth culture, is still open to question.

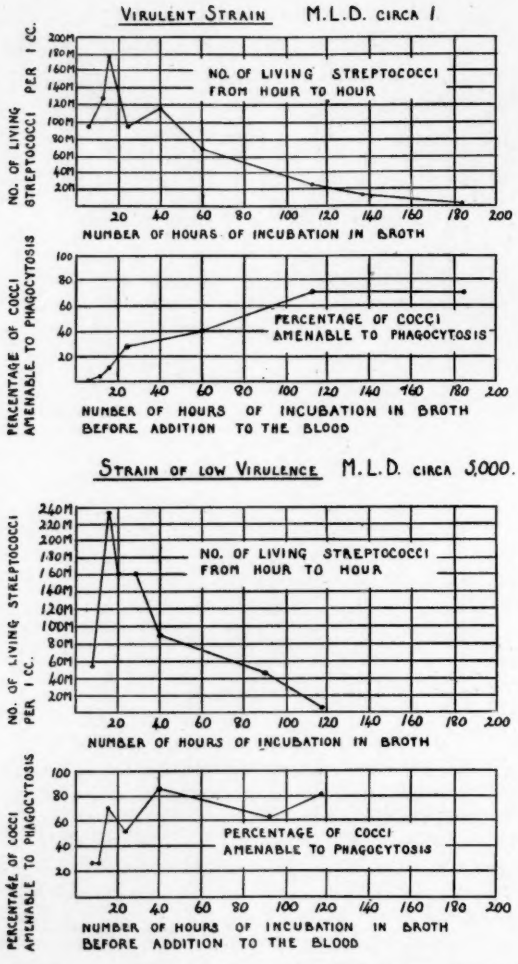
VIRULENT STRAIN M.L.D. CIRCA 1
NO. OF LIVING STREPTOCOCCI PER 1 CC.
NO. OF LIVING STREPTOCOCCI FROM HOUR TO HOUR
NUMBER OF HOURS OF INCUBATION IN BROTH
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
NUMBER OF HOURS OF INCUBATION IN BROTH BEFORE ADDITION TO THE BLOOD
STRAIN OF LOW VIRULENCE M.L.D. CIRCA 5,000.
NO. OF LIVING STREPTOCOCCI PER 1 CC.
NO. OF LIVING STREPTOCOCCI FROM HOUR TO HOUR
NUMBER OF HOURS OF INCUBATION IN BROTH
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
PERCENTAGE OF COCCI AMENABLE TO PHAGOCYTOSIS
NUMBER OF HOURS OF INCUBATION IN BROTH BEFORE ADDITION TO THE BLOOD

CHART 2. TO SHOW VARIATION IN RESISTANCE TO PHAGOCYTOSIS DURING GROWTH OF VIRULENT AND LESS VIRULENT VARIANTS OF STRAIN A. IN BROTH

It can, however, be shown that actively dividing cocci which are resistant to phagocytosis, immersed in the fluid portion of an old culture, cease growth

at once and the numbers remain stationary for 2–4 hours.* Subsequently the cocci begin to die. Before this latter occurs, a large proportion of the cocci become amenable to phagocytosis. It would thus appear that cocci resistant to phagocytosis, submitted to the unfavourable environment afforded by the fluid of an old broth culture, lose their resistance to phagocytosis. But this occurs while they are still alive. That this should occur, renders it highly probable that the cocci in an old culture which are still alive are also losing their resistance to phagocytosis. An illustrative experiment is given here (Experiment 2.)

Experiment 2.

Preparation of cultures.—Two cultures of Strain E after 11 passages through mice were prepared.

(*a*) 156-hour 5% horse-serum broth culture.

(*b*) 16-hour 5 per cent. horse-serum broth culture.

Treatment of cultures.—*Culture* (*a*) centrifuged hard for 15 minutes and the supernatant fluid removed from the organisms.

Culture (*b*) inoculated into fresh serum and incubated for 30 minutes, diluted in saline, and the cocci thrown down by centrifugation. The cocci were suspended in saline.

The washed cocci from culture (*b*) were added (1 in 5) to (1) the supernatant fluid from culture (*a*); (2) fresh serum broth. These mixtures were incubated at 37° C.

Resistance to phagocytosis of the cocci in each mixture was ascertained from time to time by the method already described.

Number of living organisms in each mixture from time to time was found by the explantation of appropriate dilutions into melted blood agar.

Results in Chart 3.

The cocci were rapidly multiplying at the beginning of the experiment. Placed in fresh broth they continue to do so. But in the fluid of an old culture they neither multiply nor do they die; the numbers remain stationary. The cocci are resistant to phagocytosis at the start and continue in this condition in the fresh broth. But in the fluid of an old culture, they lose resistance. Now as the cocci were derived, in the first place, from a rapidly dividing culture, it is a legitimate assumption that the vast majority of the cocci seen in films are alive. The same applies to the cocci after they have been in the old culture fluid for 2½ hours. Yet 50 per cent. of these can be taken up. Hence it must be concluded that the cocci submitted to the deleterious influences which afflict the cocci in an old culture, lose resistance. But this may occur while they are still alive. It is thus probable that the surviving cocci in an old culture are losing resistance to phagocytosis.

This is confirmed, indirectly, by the results of bactericidal tests with human blood, reported in a previous paper (Hare, 1929), and of mouse virulence tests with cultures of different ages as reported by Felty and Bloomfield (1924).

* This is not due to acidity, for old 5 per cent. horse-serum broth cultures (100 hours or more) are, in my experience, generally on the alkaline side of neutrality as judged by the phenol red capillator.

It is of interest in this connection that streptococci killed by heat retain the resistance to phagocytosis they had when alive (Marchand, 1898). I have confirmed this.

The changes in the cultures responsible for this fluctuation in resistance to phagocytosis will now be discussed.

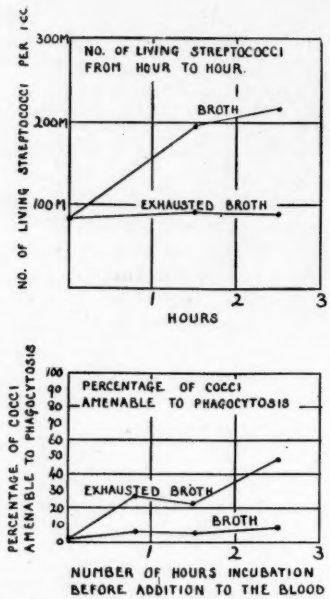

CHART 3. TO SHOW DISAPPEARANCE OF RESISTANCE TO PHAGOCYTOSIS DURING INCUBATION IN EXHAUSTED BROTH

WHAT DOES RESISTANCE TO PHAGOCYTOSIS DEPEND UPON?

The resistance of young cultures of virulent strains of hæmolytic streptococci to phagocytosis might be due to a number of factors amongst which may be mentioned:

(1) Presence in the culture fluid of some substance either harmful to leucocytes or, at least, inimical to phagocytosis.

(2) Presence of a capsule round the organism.

(3) An inability of the opsonins in the serum to opsonize the cocci.

Each of these will be discussed in turn:

(1) It was first shown by Marchand (1898) that streptococci resistant to phagocytosis, suspended in the culture fluid of streptococci which are not resistant to phagocytosis, and submitted to phagocytic tests at once, remain

resistant. The converse was also true. I have confirmed both these statements. It would thus seem that resistance to phagocytosis is an attribute of the cocci themselves and not of some substance in the fluid portion of the culture.

(2) It was thought that during the period of resistance to phagocytosis the cocci might be surrounded by a capsule; and that this might disappear with prolonged incubation. But many attempts to demonstrate such coverings have failed entirely.

(3) It was found in connection with other studies that hæmolytic streptococci resistant to phagocytosis by normal blood, digested in the serum of a patient infected with hæmolytic streptococci, and washed clear of the serum, had lost their resistance (Hare, 1928.) It was apparent, therefore, that in the serum of such a patient there was some substance which was able to abolish the resistance of these cocci to phagocytosis. Further investigation showed that this was due to the presence in the serum of such patients of a heat stable opsonin. It would thus seem that the resistance of some cultures of hæmolytic streptococci to phagocytosis in normal blood is, in reality, due to the fact that they are not opsonized by the opsonins of normal serum. This received further corroboration by the discovery that younger cultures than are usually employed (8–12 hours instead of 16–24 hours) resist phagocytosis in the presence of such serum as in that of the normal. It thus appeared probable that during incubation in a culture medium, the cocci pass through a definite cycle, so that during the period of active growth when they are most resistant of all, they cannot be opsonized by any serum, while, as the culture grows older, the cocci become more and more amenable to opsonization. The serum of the patient, therefore, differs from the normal merely in its ability to secure the phagocytosis of cultures younger than those which can be phagocyted in the presence of normal serum alone. An experiment to illustrate this is given here.

Experiment 3.

Strain C, derived originally by blood culture from a case of septicæmia following abortion. Had had three mouse passages subsequent to isolation.

Sera (*a*) Taken from the patient C— three months after leaving hospital. (*b*) Normal serum R.H. taken at the same time. Both heated at 56° C. for 30 minutes.

Method.—Cultures of various ages in 5 per cent. horse-serum broth obtained by the usual method. A specimen of fresh normal defibrinated blood was divided into two parts. To one was added 1/10 of its volume of the serum from the patient; and to the other 1/10 volume normal serum. The resistance of the cultures to phagocytosis in each specimen of blood was found by the method described.

Results in Chart 4.

Comment on this experiment.—It is evident from the curves that the presence of 1/10 heated patient's serum does make a considerable difference in the ability of the normal blood in which it is incorporated to take up the organisms. But it is noteworthy that this is particularly manifest during the stationary and early part of the decline phases of the culture. During this period, the cocci are still largely resistant to phagocytosis in the presence of normal serum

alone, but the serum of the patient is able to secure a considerable amount of phagocytosis. But the cocci in the logarithmic phase do not behave like this. They resist phagocytosis in the presence of the patient's serum as well as in that of the normal. Such cocci, in virtue of their state of active growth, are most resistant of all. The failure of the powerful opsonins of the patient to bring about their phagocytosis is added evidence in favour of the view that resistance to phagocytosis connotes failure of opsonization. For although the serum of such patients is able to bring about the opsonization and, therefore, phagocytosis of cultures younger than those which can be phagocyted in the

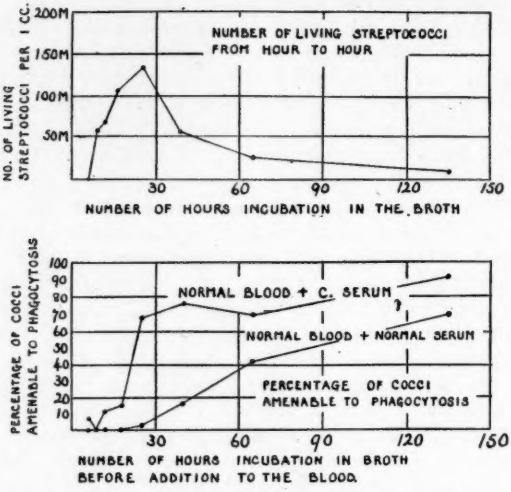

CHART 4. TO SHOW THE EFFECT OF THE PRESENCE OF HEAT STABLE OPSONINS ON THE AMENABILITY OF HAEMOLYTIC STREPTOCOCCI TO PHAGOCYTOSIS

presence of normal serum, it is inoperative on the youngest and for this reason, most resistant cultures of all.

It would thus seem that resistance to phagocytosis in normal blood is due to the inability of the opsonins in the serum to opsonize the organisms.

DISCUSSION.

The experiments quoted in this paper show that strains of hæmolytic streptococci which are virulent for animals become resistant to phagocytosis during the phase of active growth. And for reasons given, it is probable that this is, in reality, resistance to opsonization by the opsonins of the serum of the blood in which the tests are carried out. The resistance to phagocytosis which accompanies the phase of growth gradually disappears as the culture grows older, but it is not until many hours have elapsed that it finally goes.

Strains of medium virulence are not so resistant during their phase of growth, and this resistance quickly disappears altogether. Strains of minimal virulence do not become resistant at any period. The virulent strain is, then, characterized by an ability to propagate resistant cocci and to keep them resistant until the culture has gone far into the phase of decline. The avirulent strains do neither of these things.

It is a legitimate assumption, in the absence of information to the contrary, that cocci already resistant to phagocytosis which find their way into the interior of an open wound or parturient uterus, will invariably produce an infection. Cultures of virulent strains must, therefore, be looked upon as being *directly* infective over a long period of their life-history. But it would be dangerous to assume the contrary ; that the streptococci in an old culture of such a strain which have lost resistance, are necessarily unable to produce an infection. They may be unable to do so at once, but given an opportunity of multiplication in the serum bathing the abraded surfaces, they may beget resistant cocci (Hare, 1929), and thus, *secondarily*, produce an infection. It is necessary to add, however, that this applies more particularly to " middle-aged " cultures, and that the cocci from very old cultures of such strains may have quite lost the ability to bring forth resistant cocci in serum. The streptococci from such a very old culture are, for this reason, probably quite non-infective. It would thus seem that infectivity depends on two factors.

(1) The growth phase of the cocci at the time of their arrival in the tissues. This governs their resistance at that time and their ability to infect at once.

(2) The antecedents of the inoculated culture. These govern the ability of non-resistant cocci to beget resistant cocci in serum and so initiate a " delayed " infection.

What the essential features of the process may be which alter the cocci during incubation in a culture medium and so determine whether non-resistant cocci shall, or shall not propagate resistant cocci in new medium, must await further investigation.

CONCLUSIONS.

(1) Virulent strains of hæmolytic streptococci (for mice) produce in broth culture, cocci which are highly resistant to phagocytosis by human leucocytes, and which keep this resistance for many hours. Avirulent strains produce cocci which are not, at any time, resistant to phagocytosis

(2) Resistance to phagocytosis connotes failure of opsonization.

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THE LIPOLYTIC ACTIVITY OF TUBERCULAR GUINEA-PIGS' TISSUES. III.—VARIATION OF ACTIVITY WITH THE SPREAD OF THE DISEASE.

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THE statement that the tubercle bacillus is disintegrated by lipase has frequently been made. Thus, Capelli (1925) claimed to have demonstrated this point, and Calmette (1928) quotes the findings of Fiessinger and Marie that the lipase from tubercular pus has a destructive action upon the capsule of the bacillus. The stable nature of the acid-fast capsular material has repeatedly been stated to endow the tubercle bacillus with its power of resistance against disintegration by the body-cells and fluids. Bulloch and McLeod (1904) not only showed that the tubercle bacilli contained a high fatty content from which oleic, isocetinic, myristic and lauric acids were probably obtained, but that there was in addition a waxy substance which was proved to be a higher alcohol, and which was probably associated with the acid-fast nature of the organism. If we suppose that this acid-fast higher alcohol is combined in ester form with one or more of the fatty acids isolated, then it might be that the disintegration of the bacillus could be effected through the body lipases.

The constant association of a mononuclear response in T.B. infections which are not in the acute early stages, in contrast to the polymorphonuclear reaction towards infections of a more acute kind, is of interest, since Fiessinger and Marie (1909) have shown that whilst lymphoid tissue and lymphocytic exudates are rich in esterases, polymorphonuclear exudates are devoid of them. It seems to us that the open nature of the question whether or not there is any relationship between the lipolytic activity of the tissues and the resistance of an animal to progressive infection with the tubercle bacillus was worthy of further study, and that by using highly susceptible animals and artificially infecting them with tubercle bacilli, we might be able to observe whether or not there was any increased lipolytic activity in the early stages in those organs which are directly attacked.

Preliminary observations (1927) which had been carried out using ethyl butyrate as substrate suggested that, whilst the lipolytic activity of the livers, spleens, hearts and kidneys of guinea-pigs which had been infected with tubercle for a period of six weeks was markedly reduced in activity when compared with those of normal animals, the lung, a later seat of infection, gave results which in no way differed from the normal. The heart and kidney tissues were not,

of course, specifically infected with tubercle formation, so that their decreased activity might be accounted for by a general toxæmic effect, and if this were so the normality of the lung results might depend on an increased activity of that organ. These results suggested the desirability of extended studies along these lines.

EXPERIMENTAL.

In the following investigation the animals used were invariably killed by bleeding, and the livers, lungs and kidneys removed and freed from gross fat; in the case of the lung, care was taken to avoid including the tracheo-bronchial lymph-nodes. The tissues were placed in water under toluene, and transferred to the Royal Technical College, Salford. Within 4 hours of their removal from the animal body, the tissues were ground up with sand, washed into a suitable bottle with water, and glycerine added to make a 50 per cent. glycerine water mixture. The bottles were stoppered and left standing in the ice-chest for 48 hours in order to obtain an adequate extraction of the tissue esterase. The quantities of the glycerine-water mixture which were found to be suitable were 1–1½ litres for the liver tissue, and 300–400 c.c. for the lungs and kidneys, according to the approximate weight of the tissue. These quantities were found adequate for the extraction of about 98 per cent. of the esterase affecting the hydrolysis of ethyl butyrate. The extract was strained through a straining cloth fitted into a Buchner funnel and thoroughly washed through; the filtrate and washings were made up to a suitable volume with distilled water, placed under toluene, tightly stoppered, and kept in the ice-chest until required.

Three 50 c.c. flat-bottomed Jena flasks were taken, one as reaction vessel and the other two as controls. To the reaction flasks were added 10 c.c. of Clarke's phosphate buffer pH 8, a suitable volume of extract, and of 0·2 per cent. phenol red. To the control vessels were added (1) 10 c.c. phosphate buffer pH 7·6, a volume of boiled enzyme extract equal to that used in the experimental flask, the same amount of phenol red, and 0·2 c.c. of ethyl butyrate. (2) 10 c.c. phosphate buffer pH 7·2, boiled enzyme, phenol red and ethyl butyrate as in (1). The three flasks, tightly stoppered, were immersed in the thermostat at 37·5° C., and when the contents of the flasks had reached the temperature of the bath, 0·2 c.c. of ethyl butyrate was added to the reaction vessel. The flasks were shaken every half minute, and the times taken for the reaction vessel to reach pH 7·6 and pH 7·2 noted. Comparisons were always made under a standard daylight lamp. For comparative results the total time taken to complete the reaction pH 8·0 to pH 7·2 was used. Duplicate readings were always taken, and usually agreed to within half a minute. The mean of the two values is the one presented in the following tables. It was also decided to base the lipolytic activity of a tissue on the basis of the relative power of fixed amounts of nitrogenous material rather than the previous method of working on a fixed weight of tissue. This assumes that the enzyme is associated with the tissue protein, and reduces the error due to variations in the non-nitrogenous constituents of the tissue used. The nitrogen determinations were carried out by Kjeldahl's method on 25 c.c. of the extract containing about 0·004–0·008 gm. of nitrogen. Duplicate readings were

always carried out and usually showed no variation. The reaction times were then calculated on 0·0005 gm. N in the case of the liver, and 0·005 gm. N for the lung and kidney tissues. The activity of the former is about ten times that of the latter.

The first point which was studied was the relative activity of the non-functioning caseous tissue and the remaining functioning tissue of the organ. For this purpose the liver of a cow, which was infected with tubercle, was taken, and the well-defined tubercles were carefully dissected away from masses of apparently uninfected tissue. Both types of tissue were taken and compared on the old weight of tissue basis. The caseous material in 0·18 gm. aliquot portions gave quite a negligible reaction, and the amount of buffer normally added in the test had to be reduced to a tenth of that normally present in order to obtain a reaction at all comparable with that obtained with 0·12 aliquot portions of the dissected functioning tissue. These results indicated that a more careful interpretation of the significance of our previous studies was desirable, and a wider investigation should be attempted. They showed that the lipolytic activity of the functioning tissue of the infected spleens, livers and lungs would in all cases be much greater than the results obtained for the whole organ, and in addition indicated a probable hyper-activity of the lung tissue, in contrast with the hypo-activity of the non-locally infected heart and kidney tissues. As a preliminary to a more extended investigation, we proceeded to determine the normal variation in the activity of the tissue of young adult male, female, and pregnant female guinea-pigs. The following reaction times were obtained :

9 *Males :*

Liver	per	0·0005	gm. N.	Average time	13·8	mins.	Variation	11·7	—16·2	mins.
Lung	,,	0·005	,,	,,	12·5	,,	,,	9·4	—16·1	,,
Kidney	,,	0·005	,,	,,	11·5	,,	,,	8·7	—14·4	,,

12 *Females :*

Liver	per	0·0005	gm. N.	Average time	13·1	mins.	Variation	9·9	—16·2	mins.
Lung	,,	0·005	,,	,,	12·8	,,	,,	9·2	—15·9	,,
Kidney	,,	0·005	,,	,,	12·5	,,	,,	8·5	—16·0	,,

5 *Pregnant Females :*

Liver	per	0·0005	gm. N.	Average time	12·8	mins.	Variation	11·0	—16·2	mins.
Lung	,,	0·005	,,	,,	12·3	,,	,,	10·5	—14·6	,,
Kidney	,,	0·005	,,	,,	12·5	,,	,,	10·9	—15·6	,,

These results indicate that there is no pronounced difference in the esterase activity of liver, lung and kidney tissues of male, female, or pregnant animals.

THE EFFECT OF IRRADIATION WITH A MERCURY VAPOUR LAMP.

The suggestion has often been made that heliotherapy, especially in non-pulmonary cases of tuberculosis, was extremely beneficient, and more lately that the use of ultra-violet light is even better, since its action can be more

readily controlled (Watson, 1929). Sonne (1929), however, showed that the irradiation of guinea-pigs artificially infected with the tubercle bacillus gave no reliable results. It seemed of interest to determine the effect of such irradiation on the esterase activity of normal guinea-pigs. Two dozen guinea-pigs were taken and were kept in the dark. Half were irradiated twice weekly for ten minutes with a mercury vapour lamp. One animal from each group was killed at weekly intervals, commencing two weeks after the first irradiation, and the esterase activity of the liver, lung and kidney tissues determined. The results can be summarized as follows :

Reaction Times Irradiated Animals :

Livers	per	0·0005	gm. N.	Average time	13·3	mins.	Variation	11·0—16·2	mins.
Lungs	,,	0·005	,,	,,	13·3	,,	,,	9·7—18·0	,,
Kidneys	,,	0·005	,,	,,	12·2	,,	,,	9·5—15·8	,,

Reaction Times Non-irradiated Animals :

Livers	per	0·0005	gm. N.	Average time	11·6	mins.	Variation	9·9—13·9	mins.
Lungs	,,	0·005	,,	,,	11·9	,,	,,	8·6—15·9	,,
Kidneys	,,	0·005	,,	,,	11·6	,,	,,	8·5—15·3	,,

No progressive changes in the activity of the tissues could be detected in either series, but the results suggest that the esterase activity of the non-irradiated guinea-pig tissues is greater than that of the irradiated and less subject to variation. It may also be noticed that the non-irradiated animals which had been kept in the dark showed a greater esterase activity for the tissues studied than the 26 previously used which had been kept in open pens.

THE ACTIVITY OF GUINEA-PIG TISSUES INFECTED WITH TUBERCLE BACILLI.

Guinea-pigs were infected with 6-week cultures of a strain of the tubercle bacillus of human origin grown on Dorset's egg medium. The cultures were suspended and ground up in saline and injected into the groin of the animal. Animals were killed by bleeding at periods varying from 12 days to 7 weeks. The following results were obtained :

TABLE.—*Results with Animals Infected with Tuberculosis.*

Series.	Sex.	Period of infection.	Reaction time in minutes. Liver per 0·0005 gm.	Lung per 0·005 gm.	Kidneys per 0·005 gm.
I. Local lesions in glands	M.	12 days	17·7	11·5	11·5
	M.	12 ,,	17·0	12·0	10·0
	M.	19 ,,	..	12·4	15·5
	M.	19 ,,	18·0	14·4	11·0
II. Spleen slightly affected	M.	18 days	20·3	14·1	11·3
	F.	10 ,,	27·0	14·3	14·5

TABLE.—*Continued.*

Series.	Sex.	Period of infection.	Reaction time in minutes. Liver per 0·0005 gm.	Lung per 0·005 gm.	Kidneys per 0·005 gm.
III. Liver slightly affected	F.	21 days	23·5	8·6	15·1
	F.	10 ,,	22·0	9·9	16·5
	M.	6 ,,	15·4	9·9	11·4
	M.	19 ,,	17·2	11·1	12·8
	M.	20 ,,	16·4	9·0	10·9
	M.	20 ,,	17·1	9·8	..
	M.	19 ,,	15·8	12·5	11·8
	M.	18 ,,	14·9	12·8	11·3
	M.	19 ,,	14·1	8·5	11·1
	M.	19 ,,	13·6	10·0	..
	F.	21 ,, (pregnant)	17·6	7·2	13·3
		Average times	15·2	9·95	12·4
IV. Liver heavily affected	M.	21 days	23·0	9·5	17·9
	M.	25 ,,	20·0	10·2	16·1
	M.	35 ,,	15·2	11·9	13·2
	M.	35 ,,	17·7	7·4	16·8
	F.	35 ,,	25·7	10·2	17·5
	F.	35 ,,	27·3	13·3	18·1
		Average times	21·5	10·4	16·4
V. Lung slightly affected	M.	25 days	20·8	7·6	21·7
	M.	25 ,,	23·5	9·2	14·0
	M.	35 ,,	24·1	..	13·2
	F.	49 ,,	Indeterminate	9·8	18·4
	M.*	49 days	14·5	7·3	11·4
		Average times	20·7	8·5	15·7

* Local T.B. lesion, glands and spleen heavily infected. Lung shows early tubercle formation. The liver showed no signs of tubercle formation and on histological examination appeared quite normal.

SERIES VI.—*Animals only infected on 2nd heavy injection. The first two were killed 7 days after second inoculation. Tubercle bacilli demonstrated in local lesion, but not in glands. The third showed infection of glands and a few tubercles in spleen.*

Sex.	Period of 1st injection.	Period of 2nd injection.	Reaction time in minutes. Liver.	Lungs.	Kidneys.
F.	35 days	7 days	9·5	8·1	10·5
M.	35 ,,	7 ,,	9·2	11·8	11·0
M.	35 ,,	10 ,,	13·6	9·0	15·8

A series of 9 animals which were not infected by suspension of proved viability gave the following results :

Liver	per	0·0005	gm.	N.	Average time	14·7	mins.	Variation	11·4—18·0	mins.
Lung	,,	0·005	,,	,,	,,	10·7	,,	,,	6·7—12·7	,,
Kidneys	,,	0·005	,,	,,	,,	11·8	,,	,,	8·2—16·8	,,

In the series of 31 artificially infected animals we can at once discern certain progressive changes. In the earliest straightforward cases there is definite evidence of pronounced lipolytic hypo-activity of the liver tissue, which is, however, compensated to a marked degree in those animals killed whilst showing early tubercle formation in this organ. It is interesting to note that in the 3 animals which were only successfully infected after a second heavy inoculation, the liver reaction times are hyperactive, and that, moreover, 2 of them are more active than any of the animals (normal or infected) examined during the last three years. There is also a definite tendency for the kidney tissues to become less active as the disease progresses, and this is particularly marked once the liver has become riddled with tubercle. The most striking results are those obtained with the lungs in which there is a slight indication of lipolytic hypo-activity during the period of gland and spleen infection, but definite hyperactivity has set in by the time that the liver infection is first apparent, and increases as the general infection spreads. At a later period the lung also commences to show evidence of tubercle formation, and its hyperactivity becomes most marked. The last animal in Series 5 also deserves notice, since, contrary to the general rule, the infection had apparently failed to make headway in the liver tissue. The reaction time of the liver in this case is a normal one, as is that of the kidneys, but the lung is markedly hyperactive. Series 6 gives the results obtained with 3 animals which were only successfully infected after a second inoculation with a living T.B. suspension. Both the liver and lung tissues possess a greater lipolytic activity than do those of normal animals. Finally, we notice that 9 animals out of a total of 40 which were inoculated with viable suspensions of T.B. showed no signs of disease. The varying periods of time between the date of inoculation and the date on which these animals were killed makes deduction on these figures undesirable, and indeed the summarized results reveal nothing abnormal beyond a slight lipolytic hyperactivity of the lung-tissue.

SUMMARY AND CONCLUSIONS.

(1) The lipolytic activity of the liver, lung and kidney tissues of 26 normal guinea-pigs has been studied. There was no pronounced difference as between the sexes.

(2) A further series of 24 animals which were kept in the dark, one-half receiving ultra-violet radiation from a mercury vapour lamp, were similarly studied. No progressive change was apparent in either group as compared to the normal.

(3) A series of 40 guinea-pigs were inoculated with suspensions from the same strain of tubercle bacillus. Twenty-eight were infected at the first

attempt, and 9 showed no sign of disease at post-mortem. The infected animals were killed at different stages of the disease. There was a definite sustained decrease in the lipolytic activity of the kidney tissues as the disease spread. In the liver there was an early pronounced drop in lipolytic activity, a partial recovery during the period of early tubercle formation in the organ, and a final sinking away. The extent of this late decrease was probably more apparent than real. The lung tissues, however, showed a definite progressive increase in lipolytic activity, which still revealed an upward tendency during the period of early lung involvement.

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